An investigation of the mechanism by which NGF regulates the expression of the BH3-only protein DP5 in sympathetic neurons

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Abstract

Apoptosis occurs extensively during the development of the mammalian nervous system, during which time neurons depend on neurotrophic factors, such as nerve growth factor (NGF), for survival. This process is tightly controlled and the BCL-2 family of proteins are key regulators of neuronal apoptosis. DP5, a member of the proapoptotic BH3-only subfamily with highly restricted expression in the nervous system, is upregulated in sympathetic neurons undergoing NGF deprivation-induced apoptosis. Sympathetic neurons from *dp5*$^{-/-}$ knockout mice die more slowly after NGF deprivation and *dp5*$^{-/-}$ motor neurons have increased resistance to axotomy-induced death. DP5 is regulated at the transcriptional level and the c-Jun N-terminal kinase (JNK) pathway contributes to the induction of *dp5* after NGF withdrawal. The goal of my project was to determine the mechanism by which NGF deprivation induces *dp5* transcription in sympathetic neurons and to identify the regions of the gene and signalling pathways involved.

Firefly luciferase reporter constructs containing different regions of the *dp5* gene were tested in a microinjection assay using sympathetic neurons cultured *in vitro*. Levels of luciferase activity after NGF deprivation or following treatment with chemical inhibitors were measured using immunofluorescence or a dual luciferase assay. Three regions important for the induction of *dp5* after NGF withdrawal were identified: 1 kb of promoter, a 400 bp region of the intron conserved between the rat, mouse and human genes, and ~5 kb of 3'UTR. Sequences within the *dp5* promoter and intron were shown to be regulated by the MLK/JNK pathway. In addition, a functionally important binding site for the transcription factor E4BP4 was identified in the *dp5* promoter region. Overexpression of E4BP4 strongly repressed *dp5* promoter activity, whereas mutation of this site reduced basal promoter activity suggesting that an activator, such as a related PAR family transcription factor, might bind to this site in sympathetic neurons.

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Abbreviations

3'UTR	3' untranslated region
Aβ	β-amyloid peptide
ALS	amyotrophic lateral sclerosis
AP-1	activator protein-1
Apaf-1	apoptotic protease-activating factor-1
ARE	adenylate and uridylate-rich element
ATF	activating transcription factor
BDNF	brain-derived neurotrophic factor
BH	BCL-2 homology
bZIP	basic leucine zipper
CARD	caspase recruitment domain
ced-3/-4/-9	cell death defective-3/-4/-9
ces-1/-2	cell death specification-1/-2
CNS	central nervous system
CRE	cAMP response element
CREB	CRE-binding protein
DMSO	dimethyl sulphoxide
dn-Jun	dominant negative c-Jun
DRE	downstream regulatory element
egl-1	egg laying defective-1
EMSA	electrophoresis mobility shift assay
ERK	extracellular signal regulated kinase
FOXO	forkhead box, class O
IAP	inhibitor of apoptosis
JIP1	JNK interacting protein 1
JNK	c-Jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MKK4/7	MAPK kinase 4/7
MLK	mixed lineage kinase

NGF	nerve growth factor
NSM	neurosecretory motoneurons
ORF	open reading frame
PAR	proline- and acidic- amino acid-rich
PI3-K	phosphoinositide 3-kinase
PNS	peripheral nervous system
PS	phosphatidylserine
RT-PCR	reverse transcription polymerase chain reaction
SCG	superior cervical ganglion
TNF	tumour necrosis factor
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TRE	TPA response element
Trk	tropomyosin-related kinases

1 Introduction

1.1 Overview of apoptosis

1.1.1 History of cell death

Apoptosis is an important form of programmed cell death that plays a crucial role during embryonic development, normal tissue homeostasis and in defence against pathogens. The involvement of this and other forms of cell death in normal physiology, as well as pathology, is gaining ever increasing attention, reflected by the large number of publications each year. A literature search using PubMed for papers containing the term "apoptosis" results in the identification of over 100,000 publications to date. Despite major advances over the last 20 years in our understanding of the underlying mechanisms that are involved in cell death and the importance of this process, this field of research was widely neglected for many years following the first identification of this phenomenon over 150 years ago. Examples of cell death can be found throughout 19th century literature (reviewed in (Clarke and Clarke, 1996) and the first observations of cell death during normal vertebrate development were recorded in 1842 by Carl Vogt, who identified dying cells in developing toad embryos. By 1858, Virchow recognised that more than one form of cell death existed (Afford and Randhawa, 2000) and later the availability of histological staining allowed further advances in the identification of the morphologies associated with cell death. The first drawings of cells undergoing apoptosis, then named chromatolysis, were made by Walther Flemming in 1885 (Flemming, 1885). By 1914, enough data existed for Ludwig Gräper to publish a review proposing that chromatolysis was a mechanism to eliminate cells and counter balance mitosis, also suggesting that the broken down cell was engulfed by neighbouring sister cells (Gräper, 1914). Despite the importance of this review, it was largely ignored, possibly due to its publication in a German journal at the outbreak of the First World War. It was not until 1951 that this concept came to light again when Glücksmann recognised cell death during embryogenesis as a normal process although, unlike Gräper, he did not consider that this was the same as the death occurring in adult tissues

(Glücksmann, 1951). In the 1960s developments in electron microscopy advanced studies of cell death at the ultra-structural level during which time Kerr was able to further study the process of "shrinkage necrosis", which he had previously identified as a form of non-degenerative cell death that was distinct from necrosis in its histological appearance and occurred in the liver following ischaemic injury (reviewed in (Kerr, 2002). In 1972 Kerr and colleagues combined these results with other observations, including the identification of excessive death during normal development, and determined these deaths to be a "distinctive form of cell death with ultrastuctural features suggesting an active, inherently programmed phenomenon" (Kerr *et al.*, 1972). This was renamed "apoptosis".

Although apoptotic morphology had, by then, been well defined, the current interest in apoptosis only came after the identification of some of the biochemical and genetic processes involved (reviewed in (Vaux, 2002). In particular, the initial isolation of the genes involved in cell death in the nematode worm (Ellis and Horvitz, 1986;Horvitz *et al.*, 1982) and the identification of mammalian homologues (Hengartner and Horvitz, 1994;Yuan *et al.*, 1993) increased the interest in this field of research. Current areas of research include the further identification of genes involved in apoptosis, as well as the mechanisms by which they are regulated, to gain further understanding of how this process is controlled in response to a wide range of stimuli.

1.1.2 Defining apoptosis

In reviewing the literature, there appears to be some confusion over the terminology used to describe different forms of cell death, with phrases such as "programmed cell death" and "apoptosis" meaning different things to different people (Schwartz and Bennett, 1995;Vaux, 1999). Below is a summary of the most common usage of these terms, and a selection of some of the different classifications of cell death and how these relate to each other is shown in Figure 1.1. Broadly speaking, cell death can be divided into two branches: programmed and accidental death. The phrase "programmed cell death" was originally coined by Lockshin in 1964, who used it to describe a series of controlled events that were steps leading towards the self-destruction of a cell

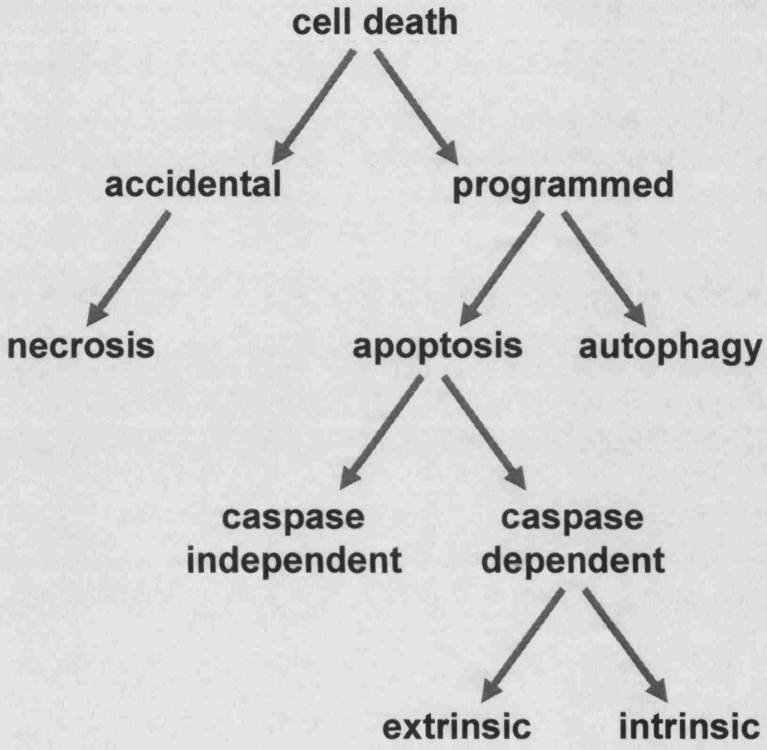


Figure 1.1 Forms of cell death

Cell deaths can be classed into different groups, depending on the mechanisms and signalling pathways involved in executing death.

(Lockshin and Williams, 1964). The idea that this is a managed process implies a form of genetic control and is perhaps better defined as cell death with a dependence on either genetically-encoded signals or activity from within the dying cell itself (Lockshin and Zakeri, 2004).

Apoptosis is one form of programmed cell death and was originally defined by its distinct morphology, observed following multiple, heterogeneous stimuli and in different cell types and species. The changes that occur during apoptosis include cytoplasmic shrinkage, chromatin condensation and margination to the inside of the nuclear membrane, plasma membrane blebbing and the formation of membrane-bound "apoptotic bodies", which give apoptosis its name (*apó* meaning from, *ptósis* meaning fall, together taken as meaning "to fall away" as leaves fall from a tree (Kerr, 1971;Kerr *et al.*, 1972). These apoptotic bodies are phagocytosed by macrophages and neighbouring cells, which engulf and digest these remains of the dying cell and prevent any leaking of the cytoplasmic contents that would otherwise elicit an inflammatory response (Henson *et al.*, 2001). This mechanism of cell death therefore allows for the highly ordered elimination of unwanted cells. In addition to these morphological changes, a number of biochemical hallmarks for apoptosis have been identified. For example, in most apoptotic cells there is increased endonuclease activity that results in the sequential cleavage of chromosomal DNA into fragments of approximately 180 base pairs, which appear as a characteristic DNA ladder in an ethidium bromide-stained gel (Williams *et al.*, 1974;Wyllie, 1980). In addition, early during apoptosis there is a loss of membrane asymmetry in which phosphatidylserine (PS), a molecule normally only found on the inner leaflet of the plasma membrane, is externalised. Exposed PS can act as a signal for phagocyte recognition and may be detected by staining with annexin V (Fadok *et al.*, 1992;Fadok *et al.*, 1998;Verhoven *et al.*, 1995).

Although apoptosis is commonly used as a synonym for programmed cell death, other forms of programmed cell death do exist but cannot always be clearly defined and distinguished. Autophagy appears to be distinct from apoptosis and is a process in which a cell degrades its own components within autophagic vacuoles, rather than degradation occurring following phagocytosis. It is likely that this form of cell death has a distinct role, emphasising the importance of defining it as a separate form of cell death

to apoptosis (Edinger and Thompson, 2004;Lockshin and Zakeri, 2004;Nelson and White, 2004).

Cell death that occurs accidentally following severe and acute injury such as abrupt anoxia and extreme physiochemical injury, is broadly defined as necrosis. This general term originally defined the morphology of dead cells after such insults (Majno and Joris, 1995) but is now often used to refer to the processes involved. In the steps leading to necrosis, the entire cytoplasm swells (known as oncosis) and there is a loss of membrane integrity. This causes the plasma membrane to rupture and the cell's contents leak out, which elicits a damaging inflammatory response that can harm the surrounding tissue (Wu *et al.*, 2001). Although in the traditional paradigm, unlike apoptosis, necrosis is generally regarded as a passive unregulated pathological process, there have been some recent suggestions that this form of death may result from an active molecular pathway, further confusing the definitions of cell death (Edinger and Thompson, 2004;Nelson and White, 2004). It is possible that apoptosis and necrosis are two extremes of a spectrum of deaths or form part of a continuum with overlapping activities (Fink and Cookson, 2005;Kanduc *et al.*, 2002).

1.1.3 Roles of apoptosis

1.1.3.1 Physiology

Apoptosis has been shown to play an important role in the normal development of organisms ranging from the nematode worm to humans, during which time cells not only undergo apoptosis to eliminate any underlying defects that arise but also apoptosis can be considered an alternative form of cell differentiation. Although defects preventing normal apoptosis during nematode development have no impact on worm survival, despite these worms having 15% more cells than normal (Ellis *et al.*, 1991b), flies deficient in apoptosis die early in development (White *et al.*, 1994), indicating the importance of this process in the development of higher organisms.

Apoptosis has a range of different functions in development. It is known to be important for the sculpting of structures, for example in digit formation, made evident by infants who are deficient in this form of cell death and are born with webbed fingers

(McCarthy, 2003). Apoptosis is also involved in the deletion of structures which have formed but are no longer required and allows a degree of flexibility for the adaptation of primordial structures for different functions at various stages of life. Deletion of these structures may be due to developmental changes, in which certain features are no longer needed, such as removal of the tadpole tail during metamorphosis (Baehrecke, 2002;McCarthy, 2003). Alternatively ancestral features that have remained throughout evolution but no longer retain a function can be removed, such as the pronephric tubules that form functioning kidneys in fish and amphibian larvae but not in mammals and so are deleted (Jacobson *et al.*, 1997;Meier *et al.*, 2000). Apoptosis also has a function in controlling cell numbers during development, a feature that is particularly apparent in the developing vertebrate nervous system, in which both neurons and oligodendrocytes are overproduced. Approximately half of these cells are eliminated by apoptosis, a process which helps ensure the correct development of a functioning nervous system (Jacobson *et al.*, 1997).

As described above, during its identification as a form of cell death, apoptosis was found to be important not only during development but also in regulating normal tissue homeostasis and it is estimated that of the 10^{14} cells in the human body as many as 10^{11} undergo apoptosis each day (Wu *et al.*, 2001). In addition to counterbalancing the effect of mitosis, apoptosis acts to remove any abnormal cells, maintaining DNA fidelity and proper cell function. Large scale apoptosis also occurs during the development and maintenance of the vertebrate immune system in which lymphocytes that bind to autoantigens are eliminated to prevent self-recognition and autoimmunity (Wu *et al.*, 2001).

1.1.3.2 Pathology

Despite having a crucial function in normal development and homeostasis, apoptosis is also associated with a number of pathologies. Apoptosis is regulated at many levels and mutations can arise that disrupt one of the stages involved in apoptosis. Conditions associated with alterations in apoptosis can generally be divided into two groups

(reviewed in Thompson, 1995): pathologies resulting from insufficient apoptosis or from excessive apoptosis.

Mutations leading to an abnormal resistance to apoptosis can result in a range of pathologies including cancer, autoimmune disease and viral infection. It was previously thought that increased cell proliferation was the important factor in the development of many cancers, but there is increasing evidence that in normal cells enhanced proliferation is often compensated by enhanced cell death (Brunner and Mueller, 2003;Hoffman and Liebermann, 1994). It is becoming increasingly apparent that tumorigenesis often arises due to a decreased ability of cells to undergo apoptosis, and oncogenes, such as *bcl-2*, an inhibitor of apoptosis, are known to contribute to the formation of some tumours (Johnstone *et al.*, 2002). Inappropriate cell survival also has consequences in diseases of the immune system since apoptosis is important for the removal of both autoreactive lymphocytes and the removal of excess cells following an immune response. Failure to do so is associated with the development of autoimmune disorders such as autoimmune lymphoproliferative syndrome (ALPS) (Brunner and Mueller, 2003). Apoptosis can be used as a defence against viruses and other intracellular pathogens, in which disruption of the cell's physiology, or detection by cytotoxic T-cells, cause the infected cell to undergo apoptosis to prevent viral propagation. Some viruses have acquired mechanisms to circumvent the host defences by blocking apoptosis. For example in adenovirus infection, the E1B 19 kDa protein, which is structurally similar to BCL-2, blocks apoptosis directly, allowing propagation of the virus (Rao *et al.*, 1992).

The above disorders are all associated with an inhibition of apoptosis but excessive cell death can also have severe implications. In many pathologies associated with premature cell death, such as myocardial infarctions and stroke, although many cells within the central ischaemic area undergo necrosis, some less badly damaged cells in the surrounding area appear to die by apoptosis (Thompson, 1995). Development of AIDS is associated with the virus-induced apoptosis of CD4+ T-cells following human immunodeficiency virus infection, which results in host immunodeficiency (Brunner and Mueller, 2003;Meyaard *et al.*, 1992;Thompson, 1995). There is also evidence that excessive apoptosis may also play a part in the depletion of specific cell populations, as

occurs during neurodegenerative diseases (see Section 1.4.2). With such a range of pathologies associated with apoptotic cell death, the importance of trying to understand the mechanisms by which apoptosis occurs, and consequently potential points of intervention that could be used for therapy, becomes apparent.

1.2 Apoptotic pathways

1.2.1 Programmed cell death in *C. elegans*

Following the identification and definition of apoptosis during normal development and homeostasis, there was relatively little interest in this area of research until some of the genes involved in controlling this process were identified in the nematode worm *Caenorhabditis elegans* (reviewed in (Liu and Hengartner, 1999). This organism has proved useful in studies of cell death because during the development of the hermaphrodite worm 131 of the 1090 somatic cells are predetermined to die and do so in a predictable manner (Ellis and Horvitz, 1986). Therefore the effect of altering gene expression on the fate of these cells can be established and the genes involved in regulating these cell deaths identified. Two genes, *ced-3* (cell death defective-3) and *ced-4*, were initially found to be essential for cell death during nematode development since loss of function mutations in either gene result in the survival of cells that would otherwise normally die (Ellis and Horvitz, 1986). These genes are negatively regulated by *ced-9* (Hengartner *et al.*, 1992) and further genetic studies have identified an additional gene, *egl-1* which is required for developmental death and which functions upstream of *ced-3*, *ced-4* and *ced-9*. *egl-1* (egg-laying defective-1) is required for the death of two hermaphrodite-specific neurons (HSN) that normally occurs in the male nematode worm. In gain-of-function mutants, these cells also undergo programmed cell death in hermaphrodite worms, whereas loss of *egl-1* function results in the survival of the 131 cells that would normally die (Conradt and Horvitz, 1998).

The proteins encoded by these genes appear to form the core execution machinery in *C. elegans* and homologues of all of these gene products have been identified in mammalian cells (see below). In the nematode worm it appears that the antiapoptotic protein CED-9 is localised mainly on the outer mitochondrial membrane

where it binds directly to CED-4 and prevents this protein from activating CED-3. To induce cell death, EGL-1 binds to and directly inhibits CED-9, which releases CED-4 and allows this to induce the proteolytic activity of CED-3 (Liu and Hengartner, 1999).

Further to this, genes upstream of the core death machinery have been identified that are important for the development of neurosecretory motoneurons (NSM). The precursors of these cells divide to form two daughter cells, only one of which survives to develop into an NSM. The sister cell undergoes cell death, a process regulated by the genes *ces-1* (cell death specification-1), which encodes a zinc finger transcription factor that protects against death, and *ces-2,* which encodes a basic leucine zipper (bZIP) transcription factor that is required for death and which appears to negatively regulate *ces-1* transcription (Ellis and Horvitz, 1991;Metzstein *et al.*, 1996). It has been proposed that *ces-1* may function to prevent NSM cell death by repressing the transcription of the proapoptotic gene *egl-1*.

1.2.2 Role of caspases

Proteases play a crucial role in regulating many cellular activities. They can exist as inactive precursors that are present at a high level, ready to act when required, and they can regulate their own activation, allowing exponential activation. Protease action is irreversible and can be highly specific, meaning that if regulated effectively, proteases can be used to govern critical biological processes (Thornberry and Lazebnik, 1998). Caspases (cysteine-dependent aspartate-specific proteases, (Alnemri *et al.*, 1996) are a family of cysteine proteases that have been implicated in both the control and execution of apoptosis. These proteases function by cleaving their substrates on the carboxyl side of specific aspartate residues and they contain a cysteine side chain that is used for catalysing peptide bond cleavage (Earnshaw *et al.*, 1999).

The first member of the caspase family to be identified was the cytokine processing enzyme interleukin-1β-converting enzyme (ICE, since renamed caspase-1). This cysteine protease cleaves the inactive 31 kDa precursor of IL-1beta, an important mediator of inflammation, to generate an active cytokine (Cerretti *et al.*, 1992;Thornberry *et al.*, 1992). Following the cloning of the *C. elegans* cell death gene

ced-3, the predicted CED-3 protein was found to have similarity to the mammalian protein ICE, which led to the proposal that CED-3 functions as a cysteine protease in the regulation of cell death in *C. elegans*, although ICE itself has no obvious role in apoptosis (Yuan *et al.*, 1993). Since this discovery, at least fourteen mammalian caspases have been identified, all of which have some similarities in amino acid sequence, structure and substrate specificity (Thornberry and Lazebnik, 1998). Each is synthesised as an inactive zymogen containing an amino-terminal prodomain, large subunit (~20 kDa) and small subunit (~10 kDa) (Kaufmann and Hengartner, 2001). Activation occurs through proteolytic cleavage at aspartate residues by autocatalysis and results in an active tetramer comprising two large and two short subunits, with both domains contributing to the active site (Earnshaw *et al.*, 1999;Thornberry and Lazebnik, 1998).

Caspases can be grouped according to their role. Caspases-1, -4, -5, -11, -12 and -14 mainly function in the processing and maturation of pro-inflammatory cytokines whereas caspases-2, -3, -6, -7, -8, -9 and -10 are involved in apoptosis. This latter group can be further divided into the effector and initiator caspases according to where they act in the apoptotic cascade (Kaufmann and Hengartner, 2001;Ryan and Salvesen, 2003). The effector (or executioner) caspases include caspase-3, -6 and -7, which function in cell disassembly (Slee *et al.*, 2001) and trigger many of the morphological changes associated with apoptosis by cleaving and consequently activating or inactivating many of the proteins involved in the execution of apoptosis. Internucleosomal DNA cleavage by endonuclease digestion is a typical feature that occurs in many cells undergoing apopotosis. The nuclease involved in this process was identified as caspase activated DNase (CAD), which is present in nonapoptotic cells in an inactive complex with the inhibitor ICAD. During apoptosis, effector caspases inactivate ICAD allowing the release and activation of CAD and consequent DNA fragmentation (Enari *et al.*, 1998). Other examples of the role of effector caspases in the execution of apoptosis include the cleavage of nuclear lamins leading to nuclear shrinkage and blebbing, gelsolin cleavage resulting in the severing of actin filaments and a loss of overall cell shape, PAK2 cleavage and activation causing active membrane blebbing and the inactivation of other

proteins involved in DNA repair, mRNA splicing and DNA replication (Earnshaw *et al.*, 1999;Hengartner, 2000;Thornberry and Lazebnik, 1998).

The effector caspases are themselves activated by proteolysis by the initiator caspases, a group which includes caspase-2, -8, -9 and -10. These caspases have longer pro-domains, which contain protein-protein interaction motifs, and are primarily monomers that require dimerisation for activation (Earnshaw *et al.*, 1999;Kaufmann and Hengartner, 2001). They function to initiate a caspase signalling cascade in response to proapoptotic signals and directly activate effector caspases. There are two well characterised pathways that trigger initiator caspase activation in response to apoptotic stimuli: the extrinsic death receptor pathway and the intrinsic mitochondrial pathway (Figure 1.2).

1.2.3 Extrinsic death receptor pathway

The extrinsic pathway of apoptosis transmits extracellular apoptotic stimuli that are initiated by specific death ligands into signals that eventually lead to the activation of a caspase cascade and apoptosis. These external signals are translated via cell surface death receptors, such as Fas (also called ApoI and CD95), which form part of the tumour necrosis (TNF) receptor superfamily. These transmembrane proteins share similar cysteine-rich extracellular domains but, in addition to this, members of the death receptor branch of this family contain a homologous cytoplasmic sequence, the death domain, which is required for apoptotic signalling (Ashkenazi and Dixit, 1998). The ligands that bind to these receptors, such as the Fas ligand (FasL), are structurally related molecules belonging to the TNF gene superfamily (Budihardjo *et al.*, 1999). The engagement of ligands to their specific cell surface receptors generally results in the transmission of apoptotic signals through the induction of receptor trimerisation, the recruitment of receptor-associated proteins and the initiation of caspase activation (reviewed in (Ashkenazi and Dixit, 1998;Budihardjo *et al.*, 1999). In the case of the receptor Fas, binding of FasL results in receptor trimerisation and the recruitment of the Fas-associated adaptor protein FADD. FADD interacts with the Fas death domain via a death domain within its own C-terminus to form the death inducing signalling complex

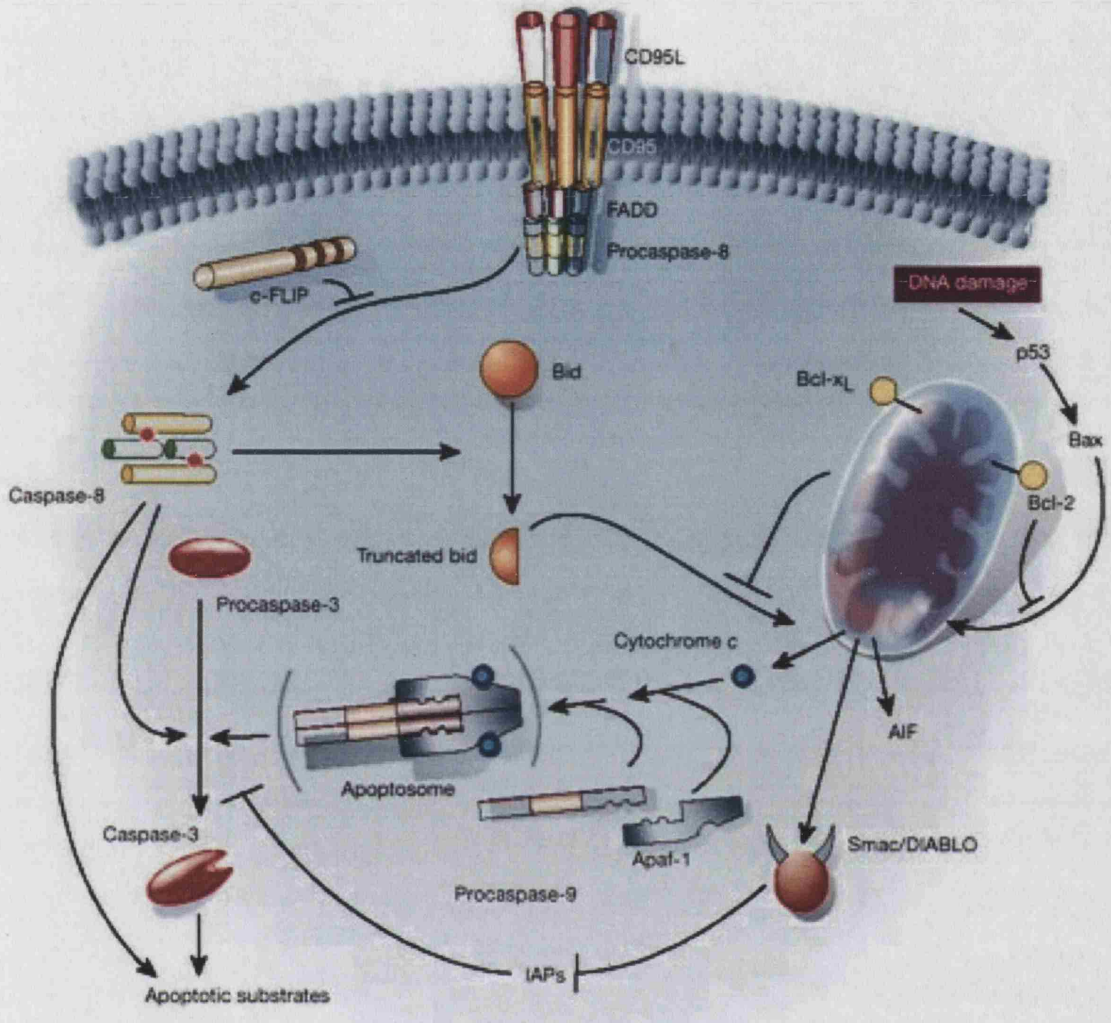


Figure 1.2 Two major mammalian pathways of apoptosis

The extrinsic pathway is triggered by members of the death receptor superfamily and involves receptor trimerization and the formation of a death-inducing signalling complex. Procaspase-8 is recruited via the adaptor Fas-associated death domain (FADD) protein, triggering caspase-8 activation and a downstream caspase cascade. The intrinsic mitochondrial pathway responds to extracellular signals as well as internal insults, the responses converging at the mitochondria and resulting in the release of cytochrome c. This leads to the formation of the apoptosome and caspase-9 activation. (Taken from Hengartner, 2000)

(DISC). FADD also contains a death effector domain (DED) within the N-terminus and this can bind to an analogous domain in procaspase-8 (Thornberry and Lazebnik, 1998). The induced proximity model of caspase activation proposes that the recruitment of several procaspase-8 molecules to the DISC increases the local zymogen concentration. These proenzymes may have a low intrinsic level of protease activity, which allows them to undergo autocatalytic cleavage and once activated they can in turn activate downstream effector caspases (Kaufmann and Hengartner, 2001;Salvesen and Dixit, 1999). Activation can occur through either the direct cleavage of effector caspases or the action of the BCL-2 family member BID. This protein is cleaved by caspases activated in the extrinsic pathway. The resultant carboxyl-terminal truncated BID (tBID) translocates to the mitochondria where it can activate the mitochondrial intrinsic pathway (Luo *et al.*, 1998).

1.2.4 Intrinsic mitochondrial pathway

The intrinsic pathway of apoptosis is mediated by the mitochondria. These organelles are integrators of cell death, allowing the convergence of both regulatory and effector molecules that bring about apoptosis in response to a range of stimuli. This generally occurs through the activation of the intiator protease caspase-9. The intrinsic pathway was first identified in experiments performed on cell extracts from normal cells, in which the addition of ATP, or preferably dATP, initiated apoptosis, evident by caspase-3 activation and DNA fragmentation (Liu *et al.*, 1996). The importance of mitochondria in this pathway was first indicated by the identification of the mitochondrial protein cytochrome c as an essential component of this pathway (Liu *et al.*, 1996). Cytochrome c is normally found loosely associated with the mitochondrial inner membrane where it functions as an essential component of the electron transfer chain, transporting electrons between complex III and IV in mitochondrial respiration (Schagger, 2001). Although cytochrome c knockout mice die during embryonic development, cells derived from early embryos and cultured under conditions to compensate for their respiratory defect fail to activate procaspase-9 and are resistant to death induced by a number of stimuli,

including UV radiation, the protein kinase inhibitor staurosporine, and serum withdrawal (Li *et al.*, 2000).

In response to the appropriate apoptotic stimuli, cytochrome c is released from the mitochondria into the cytoplasm, where it interacts with the cytosolic scaffold protein Apaf-1 (apoptotic protease-activating factor-1) (Li *et al.*, 1997). This 130 kDa protein is a mammalian homologue of the *C. elegans* death protein CED-4 and these proteins share ~50% similarity in the primary amino acid sequence of their central domain, particularly within a region containing a nucleotide binding site (Zou *et al.*, 1997). Apaf-1 also contains multiple WD40 repeats that mediate protein-protein interactions at the C-terminus and a caspase recruitment domain (CARD) at the N-terminus, which can interact with procaspase-9 (Budihardjo *et al.*, 1999). Apaf-1 alone binds dATP/ATP poorly but its affinity for this nucleotide increases approximately 10-fold following its interaction with cytochome c (Jiang and Wang, 2000;Wang, 2001). After nucleotide binding, the Apaf-1/cytochrome c/dATP complex undergoes oligomerisation to form a multimeric structure known as the apoptosome (Zou *et al.*, 1999). It appears that it is the cytochrome c-induced dATP/ATP binding rather than hydrolysis that is the important step for apoptosome formation since neither this nor caspase activation were prevented in experiments using a non-hydrolysable ATP analogue (Jiang and Wang, 2000). Following apoptosome formation, exposure of the Apaf-1 CARD sequence allows the recruitment of procaspase-9, which interacts with Apaf-1 via the CARD region in its prodomain (Wang, 2001). The apoptosome is known to have a wheel-like structure with 7-fold symmetry and procaspase-9 binds to the Apaf-1 CARD at the central hub in a 1:1 ratio (Acehan *et al.*, 2002). This high local concentration of procaspase-9 is thought to induce autoactivation by facilitating the recruitment of monomers from solution to form active dimers. Activated caspase-9 remains bound to the apoptosome where it can function to cleave and activate the downstream effector caspases (Acehan *et al.*, 2002).

1.2.5 Caspase inhibitors

In addition to the release of cytochrome c from the mitochondria during apoptosis, other factors are released that are known to affect caspase activation. A broadly distributed family of proteins, known as the inhibitors of apoptosis (IAP), normally function to inhibit caspase activity (reviewed by (Deveraux and Reed, 1999). These proteins were initially identified in baculoviruses (Clem and Miller, 1994;Crook *et al.*, 1993) but homologues have since been discovered in *Drosophila* (DIAP-1 and DIAP-2) and mammals (including cIAP-1, cIAP-2, XIAP and survivin). Cells in which IAPs are overexpressed are resistant to apoptosis induced by a wide range of stimuli (Deveraux *et al.*, 1998). The defining characteristic of these proteins is the presence of between 1 and 3 baculovirus IAP repeats (BIR) at the N-terminus. These BIR domains are motifs of ~70 amino acids, with a conserved arrangement of cysteine or histidine residues (Budihardjo *et al.*, 1999). Many IAPs also contain a RING finger zinc-binding domain towards the C-termininus, which can function as a ubiquitin ligase (Huang *et al.*, 2000;Yang *et al.*, 2000). XIAP is the best characterised IAP and has been shown to be an effective and specific direct inhibitor of caspases-3, -7 and-9 (Deveraux *et al.*, 1997;Deveraux and Reed, 1999). It is likely that, rather than protecting cells from a direct suicide hit, these proteins act as a safety mechanism to ensure minimal apoptosis due to any transient or incidental cytochrome c leakage from the mitochondria (Hengartner, 2000;Wang, 2001). These caspase inhibitors are themselves inhibited by proteins normally sequestered in the mitochondrial intermembrane space but which are released following an apoptotic signal. The proteins have been identified in *Drosophila* (HID, REAPER, GRIM) and mammals (SMAC/DIABLO, OMI/HtrA2) and function by binding and directly inactivating IAPs to free caspases (Hengartner, 2000;Meier *et al.*, 2000). SMAC is a nuclear-encoded protein containing an N-terminal mitochondrial targeting sequence that is removed on import to the mitochondria (Du *et al.*, 2000;Verhagen *et al.*, 2000). This cleavage generates a new N-terminus containing four amino acid residues, including an exposed alanine, that are required for binding to IAPs (Chai *et al.*, 2000;Liu *et al.*, 2000). SMAC binds to XIAP at the BIR3 domain via these 4 amino acids, which is similar to the XIAP-binding site in active caspase-9. Therefore

caspase-9-binding to XIAP is likely to be out-competed by increased levels of SMAC following its release from the mitochondria, relieving caspase inhibition. Consequently these proteins provide a means of rapidly relieving caspase inhibition after an apoptotic stimulus.

1.2.6 Caspase-independent apoptosis

Although caspase activation appears to be a key process in the transduction and activation of apoptosis, there is increasing evidence for the importance of death pathways in which caspases do not play a role. In addition to cytochrome c, during apoptosis other proteins are released from the mitochondrial intermembrane space, some of which appear to be involved in these caspase-independent pathways that lead to apoptosis. One such protein is apoptosis-inducing factor (AIF), which is a highly conserved flavoprotein that resembles bacterial oxidoreductase. The mature 57 kDa protein resides in the intermembrane space of the mitochondria and it has been suggested that it may have electron transfer activity (Susin *et al.*, 1999). Following the induction of apoptosis, AIF is released from the mitochondria and translocates to the nucleus where it triggers chromatin condensation and large-scale (~50 kb) DNA fragmentation. Neither of these two apoptotic effects are prevented by the caspase inhibitor zVAD-fmk, suggesting that this action is independent of caspase activity (Susin *et al.*, 1999). The functional importance of this protein is indicated by the phenotype of AIF knockout mice, which die during embryonic development, showing defects in embryoid body cavitation (Joza *et al.*, 2001). EndoG, a sequence-unspecific nuclease, is also released from the mitochondria following certain apoptotic stimuli. Although this protein normally resides in the mitochondria where it is thought to function in mitochondrial DNA replication, following its release from the mitochondria it induces the caspase-independent fragmentation of nuclear DNA (Li *et al.*, 2001). The identification of such apoptotic proteins, which also appear to potentially function in cell survival, indicates the complexity of the signalling pathways that lead to apoptosis.

1.3 Regulation of the mitochondrial pathway

1.3.1 BCL-2 family

BCL-2 is the founder member of a family of proteins that have been shown to be key regulators of many signals that lead to caspase activation and apoptosis and which play an important role in regulating the mitochondrial signalling pathway. The *bcl-2* proto-oncogene was originally identified as being involved in the t(14;18) translocation in human B-cell lymphomas and was found to permit the survival of cytokine-dependent hematopoietic cells in the absence of cytokine (Tsujimoto *et al.*, 1984;Vaux *et al.*, 1988). This mammalian protein has since been shown to be homologous to the *C. elegans* survival protein CED-9, sharing 23% sequence identity and similar death inhibitory functions (Hengartner and Horvitz, 1994). For example, *bcl-2* overexpression can suppress apoptosis in a variety of cell types both *in vitro* and *in vivo*, whereas mice deficient in BCL-2 possess abnormalities consistent with an increase in apoptosis in specific cells (Jacobson and Evan, 1994). It appears that both CED-9 and BCL-2 function using a similar mechanism to protect cells from apoptosis since expression of human *bcl-2* in the nematode worm is able to suppress apoptosis (Vaux *et al.*, 1992).

The BCL-2 family now contains an increasing number of proteins that act to either promote or suppress cell death. All BCL-2 family members possess at least one of four conserved motifs thought to be critical for the function of these proteins (Figure 1.3). These are known as BCL-2 homology (BH) domains (BH1, BH2, BH3 and BH4) and correspond to α-helical segments of the protein (reviewed in (Adams and Cory, 1998;Fadeel *et al.*, 1999;Gross *et al.*, 1999a). This family can be broadly divided into two classes: antiapoptotic and proapoptotic members. The antiapoptotic members can inhibit apoptosis in response to a range of insults and are most similar to BCL-2 itself, BCL-X_L sharing 56% homology (Adams and Cory, 1998;Merry and Korsmeyer, 1997). Most proteins in this class contain at least BH1 and BH2 but those most like BCL-2 share sequence homology through all four BH domains (Adams & Cory, 1998). The antiapoptotic BCL-2 family members also contain a hydrophobic C-terminal domain that is thought to be involved in targeting the proteins to intracellular membranes and BCL-2 is known to reside on the cytoplasmic face of the mitochondrial outer membrane,

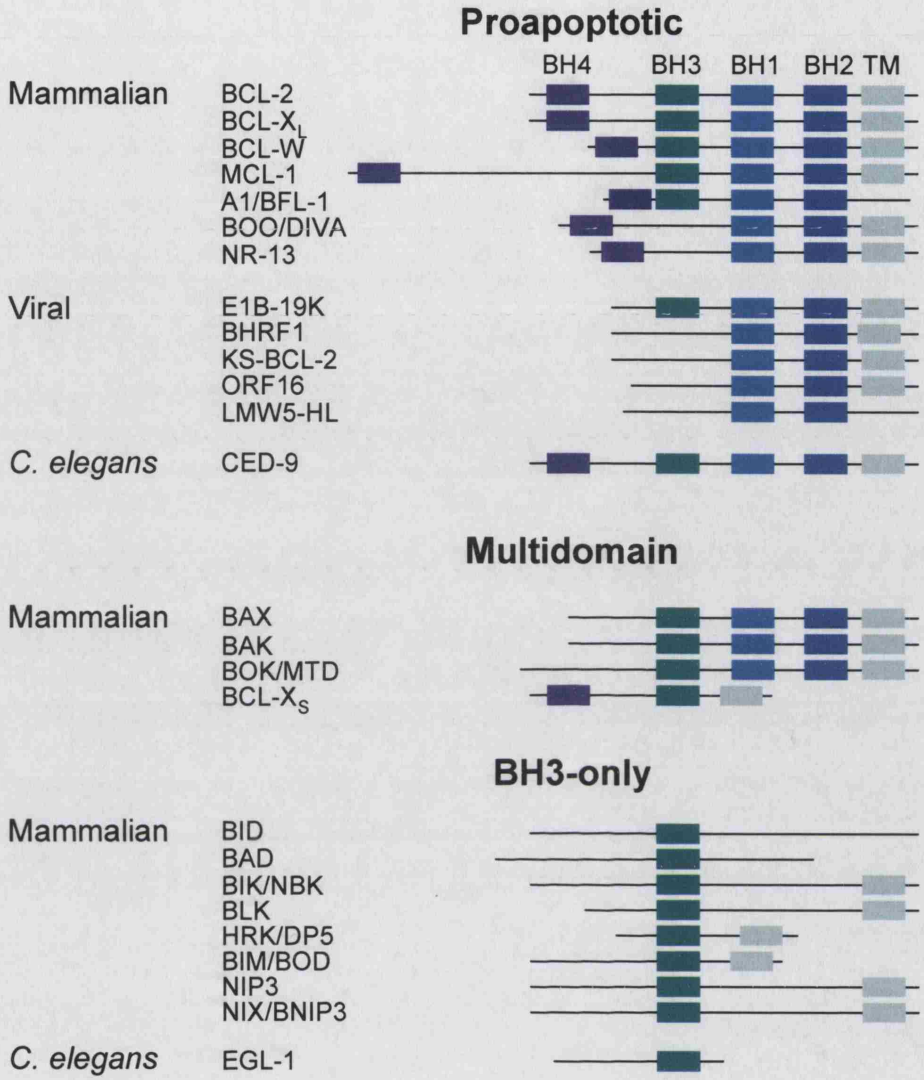


Figure 1.3. Summary of antiapoptotic and proapoptotic BCL-2 family members and their BH domains

BCL-2 family members can be grouped into different subfamilies depending on their apoptotic activity and the BCL-2 homology (BH) domains that they contain. The BH regions (BH1-4) and the carboxy-terminal hydrophobic (TM) domain of some of the identified BCL-2 family proteins are shown. (Adapted from Gross *et al.*, 1999)

the endoplasmic reticulum and nuclear envelope (Adams and Cory, 1998;Antonsson, 2001;Gross *et al.*, 1999a).

The proapoptotic members of the BCL-2 family can be further divided into two subclasses that differ in their relatedness to BCL-2. Members of the multidomain subfamily resemble BCL-2 fairly closely and include BAX, BAK and BOK that all contain BH1, BH2 and BH3 domains as well as a C-terminal hydrophobic domain (Adams and Cory, 1998;Antonsson, 2001). The other proapoptotic subfamily is the BH3-only family, whose members only share the BH3 domain and are otherwise unrelated (Figure 1.3) although some members also have a C-terminal hydrophobic domain (Adams and Cory, 1998;Antonsson, 2001).

1.3.2 Regulation of cytochrome c release

The mechanism of cytochrome c release in the absence of mitochondrial swelling appears to involve two processes: the redistribution of the cytochrome c stores in the intra-mitochondrial cristae and the mediated release of cytochrome c across the outer mitochondrial membrane. The BCL-2 family of proteins are known to be involved in controlling cytochrome c release, although exactly how these proteins function to regulate these processes is not known. Certain biochemical properties of the BCL-2 family appear to be crucial for their ability to regulate cell death. The carboxy-terminus of many family members contains a hydrophobic stretch of amino acids that comprise a transmembrane domain that targets the protein to intracellular membranes. This region appears to be important for apoptotic function since BCL-2 proteins in which this domain has been deleted show a reduction in their ability to either inhibit or promote apoptosis (Vander Heiden and Thompson, 1999). The BCL-2 proteins also have the ability to form both homo- and heterodimers with other family members, a feature that appears to be important for their role as apoptotic regulators.

The effect of different BCL-2 family members on cytochrome c release is well established. BCL-2 is an integral membrane protein that has been shown to act *in situ* on mitochondria, both in cell-free systems and intact cells, to maintain mitochondrial integrity and prevent the release of cyctochrome c from the mitochondria on which it

resides (Kluck *et al.*, 1997). Not only does BCL-2 overexpression prevent this efflux of cyctochrome c, it also prevents the initiation of apoptosis in response to a variety of death-inducing stimuli (Yang *et al.*, 1997b). Conversely, the multidomain proapoptotic BCL-2 family proteins are thought to promote mitochondrial permeabilization. The addition of BAX to purified mitochondria promotes cytochrome c release (Jurgensmeier *et al.*, 1998) and the multidomain family members appear to be crucial for apoptosis since cells isolated from BAX and BAK double deficient mice are resistant to a range of apoptotic stimuli (Lindsten *et al.*, 2000). Evidence suggests that the antiapoptotic proteins such as BCL-2 and BCL-X_L act to inhibit cytochrome c release and apoptosis by binding and sequestering the multidomain proapoptotic proteins, such as BAX and BAK, inhibiting their apoptotic effects (Adams and Cory, 1998). Heterodimers form between these two classes of protein through the interaction of the amphipathic α-helix of the BH3 domain of one protein binding to the hydrophobic groove formed by the BH1, BH2 and BH3 domain of the other. For example, mutational analysis of the BCL-2/BAX heterodimer indicates that the BH1, BH2 and BH3 domains of BCL-2 are required for heterodimer formation, and deletion of either the BH1 or BH2 domains disrupts both the interaction between these two proteins and the antiapoptotic effects of BCL-2 (Yin *et al.*, 1994). In contrast, only the BH3 domain of BAX is required for binding to BCL-2 and also the proapoptotic effect of this protein (Shangary and Johnson, 2002;Zha *et al.*, 1996a). In viable cells BAX exists as an inactive monomer located in the cytosol or loosely attached to membranes. In response to certain death stimuli, BAX translocates to the mitochondria and, following a conformational change that exposes the N-terminus, it inserts into the outer membrane as a homo-oligomerized multimer (Chan and Yu, 2004).

The exact mechanism of cytochrome c release and how this is regulated by BCL-2 family members is still unknown but several models have been proposed. BCL-2 family members are known to be able to interact with a range of proteins and so could interact with other mitochondrial outer membrane proteins to form a pore that allowed cytochrome c release. Some family members can bind to the voltage dependent anion channel (VDAC) and so could regulate the activity of this, or other channels (Green and Reed, 1998;Hengartner, 2000). It is even possible that BCL-2 family members could

form channels themselves since the antiapoptotic protein BCL-X_L has a similar structure to the pore-forming subunits of bacterial toxins such as diphtheria toxin and colicins (Lazebnik, 2001). BCL-X_L, BCL-2 and BAX can all form channels in synthetic membranes, although only BAX is able to do this at a physiological pH (Budihardjo *et al.*, 1999;Hengartner, 2000). Alternatively, the BCL-2 family could regulate cytochrome c release by inducing the rupture of the outer mitochondrial membrane, allowing cytochrome c to pass through tears in the membrane. Alterations in mitochondrial homeostasis could occur via modulations in the permeability transition pore (PTP), a large conductance channel that regulates inner mitochondrial membrane potential. Pharmacological inhibition of the PTP inhibits some forms of apoptosis, including BAX-mediated death, suggesting a possible role for this multi-protein complex in apoptosis signalling (Green and Reed, 1998;Vander Heiden and Thompson, 1999).

1.3.3 BH3-only proteins

The importance of this family of proteins was indicated by the identification of the BH3-only protein EGL-1 as a critical cell death activator during developmental apoptosis in *C. elegans* (Conradt and Horvitz, 1998). At least 11 BH3-only proteins have been identified in mammals with different expression patterns and modes of activation, reflecting the increased complexity of cell death regulation compared to the nematode. Members include BID, BIM, BIK, BAD, BLK, HRK/DP5, NIP3, NIX/BNIP3, NOXA, PUMA and MULE. All those identified to date are proapoptotic and can induce cell death when overexpressed (Puthalakath and Strasser, 2002). It has been shown that the 9-16 amino acid BH3 domain plays an essential role in the function of these proteins and deletion studies suggest that the amphipathic α-helical BH3 domain serves as a critical death domain, required for their interaction with other BCL-2 family members as well as for their killing activity (reviewed by (Kelekar and Thompson, 1998;Lutz, 2000).

Exactly how BH3-only proteins function to trigger apoptosis is still uncertain but work done using short peptides to represent the BH3 domains from different BH3-only proteins indicates that these proteins fall into two subsets that function upstream of the

other BCL-2 proteins. Some BH3-only proteins containing BID- and BIM-like BH3 domains appear to function as activators, able to directly bind to multidomain BAX and BAK. This induces oligomerisation and activation, ultimately resulting in cytochrome c release and apoptosis (Kuwana *et al.*, 2005;Letai *et al.*, 2002). Many of the BH3-only proteins bind to and inhibit the antiapoptotic members, such as BCL-2 and BCL-X_L. This derepressor class appears to neutralise the antiapoptotic proteins, sensitising the outer membrane for permeabilisation and freeing the BAX-like proteins to execute death (Kuwana *et al.*, 2005;Letai *et al.*, 2002;Moreau *et al.*, 2003). Peptides representing the effector BH3-only protein BH3 domains are potent inducers of cytochrome c release from the mitochondria in cultured cells, which may be synergistically enhanced by the presence of the derepressor subset (Kuwana *et al.*, 2005;Terradillos *et al.*, 2002). Inhibition of BCL-like proteins by this latter group alone appears to be insufficient to induce apoptosis without the presence of a direct BAX/BAK activator (Zong *et al.*, 2001).

In addition to contributing to the permeabilisation of the mitochondrial outer membrane, there is evidence that the BH3-only protein BID may contribute to the structural reorganisation of the mitochondrial cristae, making stores of cytochrome c available for release. Following treatment with p15 tBID, mitochondria were seen to undergo a BAX-independent structural remodelling with mobilisation of approximately 85% of the cytochrome c stores in the cristae making the entire cytochrome c store accessible to the outer membrane (Scorrano *et al.*, 2002). This suggests that the BH3-only proteins may have multiple functions in regulating cytochrome c release.

Until recently there had been no evidence for the involvement of the mitochondria in apoptosis in the nematode *C. elegans* but work performed by Jagasia and colleagues (2005) demonstrated that mitochondrial fragmentation occurs in *C. elegans* cells that normally undergo apoptosis during development and this fragmentation can be both induced by the BH3-only protein EGL-1 and blocked by mutation of *ced-9*, a *bcl-2* homologue. Although fragmentation occurs independently of CED-4 and CED-3 (Apaf-1 and caspase homolgues respectively), and instead appears to involve a component of the mitochondrial fission machinery, dynamin-related protein, which is necessary and sufficient for fragmentation and apoptosis in *C. elegans*, the

results suggest a role for the mitochondria in the nematode cell death pathway that involves regulation by BCL-2 family members (Jagasia *et al.*, 2005).

1.3.4 Regulation of BH3-only proteins

Due to their potent ability to initiate apoptosis, the activity of BH3-only proteins must be tightly regulated (as reviewed in (Puthalakath and Strasser, 2002). A range of posttranslational mechanisms are used to control the action of these proapoptotic proteins. Phosphorylation is known to regulate BAD as, in healthy cells, phosphorylated BAD binds to 14-3-3 scaffold proteins and so remains in the cytoplasm (Zha *et al.*, 1996b). In response to death signals, BAD is dephosphorylated allowing it to bind to and inactivate BCL-2 and BCL-X_L (Zha *et al.*, 1997). Another form of modification used in BH3-only protein regulation is cleavage. Following signalling through a death receptor, activation of caspase 8 can cleave cytosolic p22 BID at its amino-terminus. This generates a p15 carboxy-terminal fragment, truncated BID (tBID), that translocates to the mitochondria and inserts into the membrane where it induces cytochrome c release (Gross *et al.*, 1999b). After certain apoptotic stimuli, control of the BH3-only protein BIM occurs though the regulation of its intracellular localization. In viable cells BIM associates with the microtubule-associated dynein complex by binding the LC8 dynein light chain. When triggered, BIM and LC8 dissociate from the motor complex and translocate to the mitochondria where BIM is thought to interact with BCL-2 (Puthalakath *et al.*, 1999). Besides these posttranslational control mechanisms, the transcriptional regulation of some BH3-only proteins is important in apoptosis that requires new protein synthesis. In sympathetic neurons BIM is known to be regulated at this level, the mechanisms involved appearing to require the interaction of more than one pathway (see Section 1.7).

1.4 Apoptosis in the nervous system

1.4.1 Neuronal apoptosis in development

In general, neuron life history follows a process of induction, differentiation, proliferation, the formation of axons and synaptic connections, finally resulting in mature neurons with specific physiological functions. In mammals, mature neurons are among the most long-lived cells but in contrast to this, many immature neuron populations undergo extensive cell death during development. The significance of these deaths is apparent in the lethal phenotype of mutant mice deficient in some of the regulatory and executioner molecules of apoptosis. Morphological defects in these mutant mice, particularly in the central nervous system (CNS) can be seen early in embryogenesis. For example, *Apaf-1*$^{-/-}$ mutant mice show defects as early as embryonic day 9.5, suggesting a role for apoptosis in normal vertebrate development (de la Rosa and de Pablo, 2000).

There are two major populations of neuronal cells that die during nervous system development: neural precursor cells and post-mitotic neurons. Death of neural precursors occurs during the early stages of neural development and may serve a variety of functions including pattern formation and morphogenesis such as closure of the hindbrain neural tube, elimination of abnormally located or differentiated cells and controlling cell number (de la Rosa and de Pablo, 2000;Oppenheim, 1991;Sommer and Rao, 2002). During the later stages of vertebrate development there is a massive loss of post-mitotic neurons in many regions of the CNS and peripheral nervous system (PNS) where neurons are initially overproduced. Between 20-80% of these neurons undergo apoptosis over a relatively short period of time, typically during the period in which neurons form synapses with their target cells (Deshmukh and Johnson, Jr., 1997). Pioneering studies performed in the 1940s and 1950s by Viktor Hamburger and Rita Levi-Montalcini led to the theory that neuronal survival during development was directly related to the availability of survival factors produced by target cells and the identification of the prototypical neurotrophic molecule nerve growth factor (NGF) (Levi-Montalcini, 1987). Hamburger and Levi-Montalcini proposed that immature neurons compete for limited amounts of trophic factors, such as NGF, that are produced

by target cells and only those neurons that establish correct synaptic connections receive enough trophic factor support to survive (Hamburger and Levi-Montalcini, 1949). This became the foundation of what is now known as the neurotrophic theory and is believed to function to adjust the size of interconnecting neural populations, matching neurons to target cells, and eliminates improper connections (Oppenheim, 1991). Since the identification of NGF, other neurotrophins have been identified, all of which promote the survival of specific neural populations during development and include brain-derived neurotrophic factor (BDNF), neurotrophins (NT)-3, -4 and -5 (Sastry and Rao, 2000).

1.4.2 Neuronal apoptosis in pathology

Although essential during development, inappropriate apoptosis in the nervous system has been implicated in multiple neurodegenerative disorders including amyotrophic lateral sclerosis (ALS), Alzheimer's disease, ischaemia, Parkinson's disease and Huntington's disease. Much of the evidence for the potential role of apoptosis in these disorders comes from work carried out on post-mortem tissue, screened for the presence of apoptotic cells or molecules involved in the apoptotic process. For example, cleaved caspases have been detected in post-mortem tissue in all of the above diseases (Troy and Salvesen, 2002). Although useful, such studies are limited in determining the pathological processes involved since only the extent of disease at the time of death can be examined, apoptotic cells can be difficult to detect and tissue is often from patients with an advanced stage of the disease in which many neurons affected will have already been lost (Troy and Salvesen, 2002;Vila and Przedborski, 2003). Animal and cell culture models that mimic these diseases have been used to overcome some of these problems, two of which are described below.

In ALS, degeneration of the motor neurons in the spinal cord and brain occurs, which, in familial ALS, is due to mutations in the gene encoding superoxide dismutase (SOD-1) (Nijhawan *et al.*, 2000). In transgenic mice, expression of mutant forms of SOD-1 results in the development of progressive motor neuron disease, similar to the human disease. Neurodegeneration in this mouse model can be delayed by caspase inhibition or by overexpressing antiapoptotic BCL-2 indicating the involvement of

components of an apoptotic pathway in disease progression (Putcha and Johnson, Jr., 2004;Vila and Przedborski, 2003). Alzheimer's disease occurs as a result of progressive damage to neurons in the neocortex, hippocampus and basal forebrain cholinergic system, leading to loss of cognitive function (Nijhawan *et al.*, 2000;Tuszynski and Blesch, 2004). The neuropathological hallmarks of this disorder include the presence of neurofibrillary tangles composed of tau protein and/or plaques composed of β-amyloid peptide (Aβ) (Anderton, 1999). The potential role of apoptosis in this disease has been studied using Aβ to induce death. Involvement of this peptide in Alzheimer's pathology is indicated by the neurodegeneration observed in transgenic mice that overexpress Aβ (Nijhawan *et al.*, 2000). This protein has been shown to be toxic to cultured primary neurons and induces similar immediate early genes to those activated by trophic factor deprivation, suggesting the potential involvement of apoptotic genes in this process (Yuan and Yankner, 2000). Despite such evidence of apoptosis occurring in many neurodegenerative disorders, whether this process, alone or in conjunction with other mechanisms, accounts for the disease pathology, has yet to be established. If apoptosis plays an important role in disease progression, a greater understanding of the processes involved might provide potential targets for therapeutic intervention.

In addition to having a possible function in neurodegeneration, there is evidence that apoptosis is involved in the pathologies of viral infections of the CNS. Several neurotrophic viruses can induce neuronal apotosis *in vitro* which, despite causing a pathologic host response in the CNS, may function in the PNS to prevent transmission to the CNS and protect the host (Mori *et al.*, 2004).

1.5 Model systems for the study of neuronal apoptosis

1.5.1 Sympathetic neurons

The development of *in vitro* models of neuronal cell death has aided the identification of the biochemical and genetic events that occur during this process. An extensively studied model of neuronal apoptosis is that of NGF deprivation-induced death in sympathetic neurons. These cells form part of the autonomic nervous system, which functions to maintain homeostasis by regulating physiological processes such as body

temperature, cardiac output, blood pressure and blood glucose levels (Gabella, 2001). The sympathetic component of this system regulates "fight and flight" responses including increasing the heart rate, dilating the coronary arteries, vasoconstriction in the salivary glands and altering pupil size and lens curvature. These effects are counteracted by activity of the parasympathetic pathway which also innervates these targets (Thexton, 2001).

The sympathetic pathway is comprised of preganglionic neurons located within the spinal cord, the axons of which exit the spinal cord to join the spinal nerve for a short distance before reaching the sympathetic ganglia. These ganglia are collections of cells that run bilaterally and parallel to the spinal cord, the largest of which are the superior cervical ganglia (SCG) that lie at the base of the skull (Gabella, 2001). Ganglia are encapsulated by a sheath of glial cells that provide both structural and nutritional support and contain the spheroidal somata of ganglion neurons, which the preganglionic neurons form cholinergic synapses with. The ganglion neurons give rise to postganglionic axons that innervate effector organs including cardiac muscle and smooth muscle as well as endocrine and exocrine glands, in most cases forming adrenergic connections with the target tissue. Postganglionic axons from the SCG mostly travel along blood vessels to reach organs of the head as well as the upper neck (Gabella, 2001).

The events that occur during sympathetic neuron development are outlined in Figure 1.4. During development postganglionic sympathetic neurons arise from neural crest cells that have migrated ventrally from the neural tube and coalesced to form the column of sympathetic ganglia (Glebova and Ginty, 2005). Neuroblasts within the ganglia complete proliferation, undergo differentiation and begin to extend axons and dendrites. Many factors play a role in final target innervation, controlling the processes of axon initiation, proximal axon extension, growth inhibition and dendrite formation (reviewed in Glebova and Ginty, 2005). For example, the glial derived neurotrophic factor family member artemin appears to be important for axon extension since this factor can induce axon growth *in vitro* whereas mice deficient for artemin have short and misdirected axonal projections. In contrast to this, semaphorin 3F induces the collapse of sympathetic growth cones, repelling axons *in vitro* and may help promote accurate target innervation. The interaction of such factors and their precise

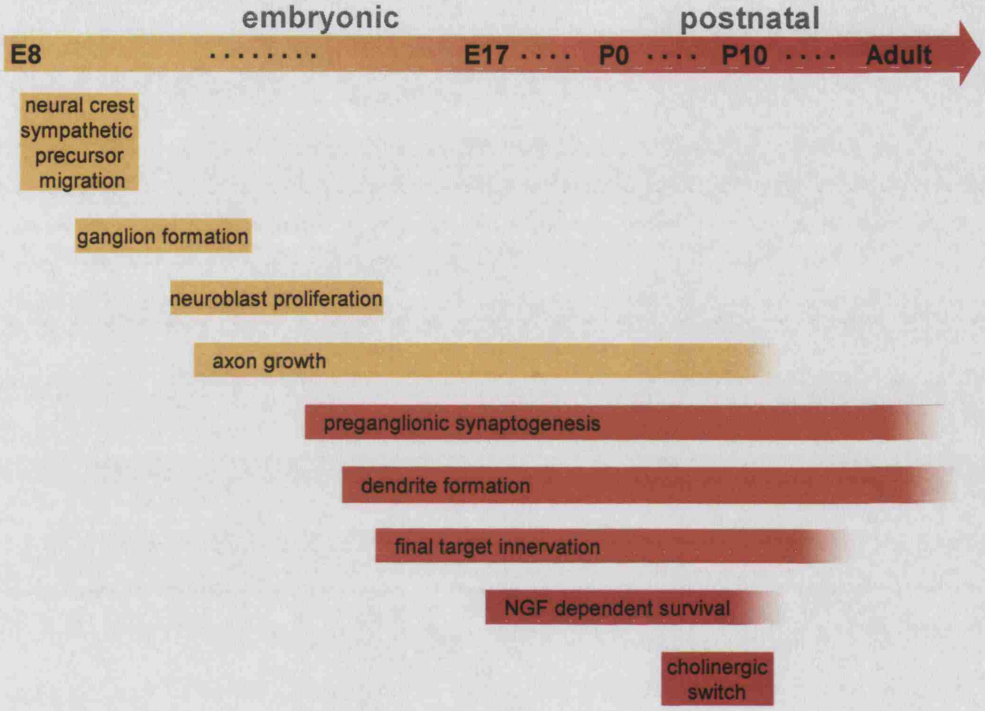


Figure 1.4 Timing of events during the development of the sympathetic nervous system in the mouse

Approximate time points are indicated. Preganglionic synaptogenesis begins early in embryonic development and dependency on NGF for survival arises at around E16 but is lost after postnatal development. (Adapted from Glebova and Ginty, 2005)

roles in neural development are still the topic of much research. In addition to axonal growth, synaptogenesis is a critical step in development of the sympathetic nervous system, since postganglionic neurons must form functional connections with both preganglionic neurons and the final target cells. Preganglionic synapses begin to develop early during embryogenesis, initially forming connections with the somata of postganglionic neurons before dendrites have formed and this can be influenced both retrogradely by target tissues as well as by presynaptic inputs. Little is known about the formation of synapses between postganglionic neurons and their targets as these connections are not classical synapses. Rather, the terminal regions of postganglionic axons are varicose and lack typical synaptic specialisation. The large varicosities contain a high concentration of neurotransmitter and may be located micrometres from the target cell, requiring efficient neurotransmitter diffusion for signal propagation (Gabella, 2001;Glebova and Ginty, 2005).

In addition to the signals required for successful sympathetic innervation during development, survival factors also play a critical role. *In vivo*, sympathetic neurons are dependent on NGF for survival from approximately embryonic-day 16 until the first week of postnatal development, during which time around 50% of these neurons die due to receiving insufficient NGF to survive (Chang *et al.*, 2002;Deshmukh and Johnson, Jr., 1997). The dependence of these peripheral nervous system neurons on NGF for survival is evident from experiments in which administration of exogenous NGF to neonatal rats caused an increase in sympathetic neuron survival. Conversely reducing the amount of NGF by either neutralization with a blocking antibody or induction of autoimmunity against NGF resulted in a decrease in the sympathetic neuron population (Deshmukh and Johnson, Jr., 1997;Jacobson *et al.*, 1997). In addition, the extensive death of sympathetic neurons can be seen in mice in which the NGF gene was deleted by homologous recombination. Although mature sympathetic neurons lose this dependence on NGF, removal of this trophic factor continues to induce similar intracellular changes to those occurring in younger cells (Pettmann and Henderson, 1998).

Developing sympathetic neurons maintained *in vitro* for 5 to 7 days in the presence of NGF reproduce this dependence on NGF for survival. In culture these cells will undergo cell death within 24-48 hours after NGF withdrawal, and this death exhibits

the classical characteristics of apoptosis, including chromatin condensation and DNA cleavage (Deshmukh and Johnson, Jr., 1997;Putcha and Johnson, Jr., 2004). Ultrastructural changes can be observed ~12 hours after NGF deprivation as the plasma membrane loses its smooth appearance and neurites start to degenerate. After 18 hours, the nuclei begin to shrink and condense, although other cell organelles remain intact until the very final stages of death (Deshmukh and Johnson, Jr., 1997;Martin *et al.*, 1988). For a period following NGF removal, cells can be rescued from apoptosis by re-addition of this trophic factor but sympathetic neurons deprived of NGF eventually lose this ability to be rescued (Chang *et al.*, 2002). This commitment point for death has been more precisely defined as the time after NGF withdrawal when only half of the neurons can be rescued by the re-addition of NGF and generally occurs around 22 hours, following NGF withdrawal. Inhibitors of both RNA and protein synthesis, such as actinomycin D and cycloheximide respectively, have been shown to block NGF-deprivation induced death, which suggests that *de novo* gene expression and protein synthesis and an active process are required for apoptosis induced by NGF withdrawal (Martin *et al.*, 1988). Neurons can only be rescued by treatment with cycloheximide up to 16 hours following NGF deprivation, a time before the irreversible commitment point, indicating that by this timepoint the required apoptotic machinery has been synthesised and the subsequent events that trigger apoptosis occur post-translationally (Deckwerth and Johnson, Jr., 1993;Deshmukh and Johnson, Jr., 1997).

The use of sympathetic neurons has a number of advantages since the cells in this model are relatively homogeneous and will undergo complete transcription-dependent death with a precise and reproducible time course. The cell death that occurs is representative of the type of death triggered by trophic factor deprivation *in vivo*, both during development or following axotomy (Deshmukh and Johnson, Jr., 1997;Pittman *et al.*, 1993). Some difficulties can arise in trying to obtain large numbers of cells and this system also lacks an efficient transfection method, although this has been partly overcome through the use of microinjection to express foreign genes (Garcia *et al.*, 1992). The development of reverse transcription-PCR has also allowed gene expression to be analysed in the small number of sympathetic neurons obtained from each animal.

1.5.2 PC6.3 cells

Some of the limitations of using primary sympathetic neurons have been overcome by use of the PC12 cell line. This cell line was derived from a rat pheochromocytoma, a tumour arising from chromaffin cells of the adrenal medulla. In the presence of serum, PC12 cells divide and resemble precursors of adrenal chromaffin cells but on the addition of NGF, these cells differentiate and take up a neuron-like phenotype. After one week in the presence of NGF, PC12 cells cease to multiply and show neurite outgrowth, similar to sympathetic neurons in primary culture (Francois *et al.*, 2001;Greene and Tischler, 1976;Vaudry *et al.*, 2002). In particular the PC6.3 subline of PC12 cells provides a good model for neuronal cell death since 90% of these cells undergo apoptosis after the removal of NGF. This death is accompanied by a loss of neurites and active membrane blebbing and occurs at a similar rate to death in sympathetic neurons following NGF deprivation. The apoptosis of PC6.3 cells, like sympathetic neurons, may be blocked by actinomycin D and cycloheximide, again suggesting a role for RNA and protein synthesis in this process. In addition to these properties, PC6.3 cells are available in large quantities and can be transiently transfected easily making this model system suitable for characterising the genetic, cellular and molecular events involved in neuronal apoptosis (Pittman *et al.*, 1993).

1.6 Apoptosis signalling in sympathetic neurons

1.6.1 Neurotrophin receptors

The effects of neurotrophins on the development and function of the nervous system are mediated by two classes of receptors that regulate the activity of intracellular signalling pathways. The high affinity tropomyosin-related kinases (Trk) receptors are a family of autophosphorylating tyrosine kinase receptors that transmit positive signals to enhance growth and survival, whereas the lower affinity p75 neurotrophin receptor ($p75^{NTR}$) is a member of the TNF-receptor superfamily that can transmit both positive and negative signals (Kaplan and Miller, 2000). Each neurotrophin can bind to and activate at least one of the Trk receptors, although each has a high affinity for a specific receptor: NGF

binds to TrkA, BDNF (brain derived neurotrophic factor) and NT-4 to TrkB and NT-3 to TrkC. TrkA has been shown to mediate most of the growth and survival effects of NGF and plays an important role in the developing nervous system since *TrkA*$^{-/-}$ knockout mice show a loss of sympathetic neurons, similar to the effect of deleting the NGF gene (Deshmukh and Johnson, Jr., 1997). The TrkA receptor is a transmembrane glycoprotein of 140 kDa that was first identified as an oncogene in colon cancer (Martin-Zanca *et al.*, 1986). This receptor contains a cytoplasmic tyrosine kinase domain and an extracellular immunoglobulin-like domain that interacts with NGF via a conserved motif shared among all neurotrophins as well as a specific motif for the interaction between NGF and TrkA (Patapoutian and Reichardt, 2001). Following the binding of NGF, TrkA dimerisation and kinase activation occurs in which the receptor phosphorylates tyrosine residues within its own cytoplasmic tail. Although the phosphorylation of three conserved tyrosines within the autophosphorylation loop of the kinase domain further activates the receptor, the other phosphotyrosines create docking sites for adaptor proteins that link TrkA receptors to the intracellular signals that coordinate neuronal survival, including the extracellular signal-regulated kinase (ERK) cascade, phospholipase C-γ1 and the phosphoinositide 3-kinase (PI3-K)/Akt pathway (Patapoutian and Reichardt, 2001).

Unlike the Trk receptors, p75^{NTR} can bind each neurotrophin and acts to either induce or prevent apoptosis, depending on the cellular context. p75^{NTR} can function, in part, to alter the affinity of Trk receptors for their cognate ligand, enhancing Trk autophosphorylation and selectivity for neurotrophins (Lee *et al.*, 2001). In addition to this, p75^{NTR} may have a more direct death-inducing role, which appears to be important for apoptosis in sympathetic neurons after trophic factor deprivation. This was demonstrated in *p75*$^{NTR-/-}$ knockout mice, in which the normal period of sympathetic neuron cell death, as well as death following NGF-deprivation in neurons cultured from these mice, is delayed (Bamji *et al.*, 1998). Results from crosses of *p75*$^{NTR-/-}$ and *trkA*$^{-/-}$ mice suggest that in the absence of p75^{NTR}, sympathetic neurons lacking TrkA are rescued from developmental death *in vivo* (Majdan *et al.*, 2001), indicating that the role of p75^{NTR} in inducing apoptosis is independent of TrkA. There is also evidence suggesting that apoptotic signalling via p75^{NTR} in sympathetic neurons involves the

binding of BDNF to this receptor (Bamji *et al.*, 1998). It therefore appears that the initiation of apoptosis signalling in this system following NGF deprivation could involve more than one mechanism.

1.6.2 Survival signalling pathways

Following Trk receptor activation the small GTP-binding protein Ras plays an important part in initiating survival signals in neurons. The involvement of this protein has been demonstrated by increasing Ras activity through the deletion of the Ras regulatory inhibitor *neurofibromatosis 1*, which prevents cell death in the absence of neurotrophins (Vogel *et al.*, 1995). Ras activation is triggered by the docking of adaptor proteins such as Grb-2 and Shc at the Trk receptor and leads to the activation of the PI3-K and ERK survival pathways. Whereas ERK has been shown to promote neuroprotection in sympathetic neurons following death induced by the antimetabolite cytosine arabinoside, PI3-K signalling promotes survival following NGF deprivation (Xue *et al.*, 2000). The importance of the PI3-K pathway in regulating neuronal survival in response to NGF has been demonstrated by use of the PI3-K inhibitor LY294002, which is a competitive inhibitor of ATP binding on PI3-K (Djordjevic and Driscoll, 2002;Yao and Cooper, 1995). Using this inhibitor, the survival of sympathetic neurons is greatly inhibited, even in the presence of NGF, and it appears that up to 80% of neurotrophin-regulated survival is dependent on PI3-K activity, although this level can vary depending on cell culture conditions (Kaplan and Miller, 2000;Pierchala *et al.*, 2004). The enzyme PI3-K is normally present in the cytosol but, following neurotrophin signalling, it is recruited to activated Trk receptors where it is activated directly or indirectly via Ras (Yuan and Yankner, 2000). The involvement of signalling by Ras is evident in experiments in which Ras inhibition has been shown to suppress NGF-mediated PI3-K activity and decreases the survival of sympathetic neurons (Kaplan and Miller, 2000). Following the docking of adaptor proteins, Ras activity results in PI3-K activation through the generation of the phosphoinositide phosphates PIP_2 and PIP_3 from the PI3-K catalytic subunit at the inner surface of the plasma membrane. These lipid products are able to recruit and activate many proteins containing pleckstrin homology domains including

phosphoinositide-dependent protein kinase-1 (PDK1), which acts together with PIP_2 and PIP_3 to phosphorylate and activate the serine/threonine kinase Akt (also called protein kinase B) (Brunet *et al.*, 2001).

Akt plays an important role in neurotrophin signalling since active Akt can support neuron survival in the absence of survival factors. Conversely, a dominant negative form of Akt inhibits survival in the presence of trophic factors (Datta *et al.*, 1999). Akt acts in part by altering gene expression through the regulation of transcription factors, including the Forkhead family, cAMP-response element-binding protein (CREB) and nuclear factor-κB (NF-κB). Phosphorylation by Akt inactivates Forkhead family members, preventing them from inducing the expression of death-inducing genes, whereas phosphorylated CREB and NF-κB promote expression of cell survival genes (Brunet *et al.*, 2001;Yuan and Yankner, 2000). In addition to altering gene expression, Akt can directly inhibit components of the apoptotic machinery. For example, Akt phosphorylates BAD (Datta *et al.*, 1997), promoting BAD association with the 14-3-3 chaperone protein and preventing its proapoptotic activity (see Section 1.3.4). These findings indicate the importance of the PI3-K/Akt pathway in promoting survival in response to NGF through several mechanisms.

As well as activating the PI3-K/Akt pathway, NGF also leads to the activation of protein kinase C (PKC) through phospholipase C-γ phosphorylation and activation. Activation of PKC has been shown to rescue sympathetic neurons from undergoing apoptosis following NGF withdrawal whereas conversely, in certain cell culture conditions, PKC inhibition results in NGF-maintained neurons undergoing apoptosis (Pierchala *et al.*, 2004). This suggests that in addition to the PI3-K pathway, signalling via PKC may be important for regulating the survival of sympathetic neurons in the presence of NGF.

1.6.3 Apoptotic signalling pathways

An important step in discovering one of the pathways involved in transmitting apoptotic signals in sympathetic neurons was the identification of the transcription factor c-Jun as a gene that was upregulated in these cells following NGF deprivation (Estus *et al.*,

1994;Ham *et al.*, 1995). Induction of *c-jun* occurs within 5-10 hours after the removal of NGF, with maximum levels of expression being observed before the cell death commitment point, between 12 and 18 hours following trophic factor deprivation (Estus *et al.*, 1994). In addition to increasing *c-jun* mRNA levels, NGF deprivation also leads to increases in phosphorylated c-Jun, the active form of the protein (Ham *et al.*, 1995). c-Jun is a member of the AP-1 family of proteins, a group of basic leucine zipper (bZIP) transcription factors, which includes the Jun, Fos, Maf and ATF subfamilies that can form both homodimers and heterodimers with each other. These recognise and bind to the specific DNA sequences 5'-TGAG/CTCA-3' (TPA response elements or TRE) and 5'-TGACGTCA-3' (cAMP response elements or CRE) to regulate the transcriptional activity of target genes (Shaulian and Karin, 2002). For example, the *c-jun* promoter contains two TRE sites, jun1 and jun2, that can bind heterodimers of c-Jun and activating transcription factor 2 (ATF-2) (Herr *et al.*, 1994), a protein that is also activated after NGF deprivation. Mutation of the jun1 and jun2 sites abolishes *c-jun* promoter activation, indicating the potential importance of these binding sites for *c-jun* expression and autoactivation (Eilers *et al.*, 1998).

A functional role for c-Jun in part of an apoptotic signalling pathway has been demonstrated in sympathetic neurons in a number of ways. Although *c-jun*$^{-/-}$ knockout mice die due to hepatic failure early during embryonic development (Hilberg *et al.*, 1993), c-Jun function has been examined by conditional inactivation of the *c-jun* gene *in vitro*, which protects sympathetic neurons from NGF withdrawal-induced death (Palmada *et al.*, 2002). In addition, inhibiting c-Jun activity by microinjecting sympathetic neurons with a dominant negative c-Jun mutant (dn-Jun) or a neutralising antibody specific for c-Jun also prevents apoptosis evoked by trophic factor deprivation (Estus *et al.*, 1994;Ham *et al.*, 1995). In contrast, overexpression of wild type c-Jun is sufficient to induce apoptosis in the presence of NGF, suggesting a critical role for this transcription factor during apoptosis in sympathetic neurons (Ham *et al.*, 1995). There is some evidence that c-Jun phosphorylation occurs in sympathetic neurons undergoing apoptosis in response to $p75^{NTR}$ activation since the neurotrophin BDNF, which triggers $p75^{NTR}$ apoptotic signalling, induces c-Jun phosphorylation coincident with apoptosis (Bamji *et al.*, 1998). Unlike the response to NGF withdrawal, apoptosis via $p75^{NTR}$

signalling does not appear to require c-Jun because apoptosis following BDNF treatment was not reduced in mice in which the *c-jun* gene had been conditionally inactivated (Palmada *et al.*, 2002).

Activation of c-Jun occurs through phophorylation by c-Jun N-terminal kinases (JNK). JNKs are serine/threonine kinases that form a subfamily of the mitogen-activated protein kinases (MAPK) and are encoded by three genes (*jnk1*, *jnk2*, and *jnk3*) with multiple splice variants (Bozyczko-Coyne *et al.*, 2001). The JNK1 and JNK2 proteins are expressed in many tissue types and although $jnk1^{-/-}$ and $jnk2^{-/-}$ single knockout mice have no obvious neuronal defects, $jnk1^{-/-}jnk2^{-/-}$ double knockout mice die during embryonic development with a pronounced neural phenotype that is due to reduced cell death in the hindbrain before neural tube closure (Kuan *et al.*, 1999). The JNK3 protein is mainly expressed in neurons and in sympathetic neurons isolated from *jnk3*-deficient mice, c-Jun phosphorylation, *c-jun* expression and apoptosis are inhibited following trophic factor deprivation, suggesting that JNK3 is important for apoptosis in this cell type (Bruckner *et al.*, 2001;Yang *et al.*, 1997a). Activated JNK translocates to the nucleus where it phosphorylates c-Jun within the N-terminal transactivation domain at serines 63 and 73, resulting in an increase in c-Jun-mediated gene transcription (Ham *et al.*, 2000). In both sympathetic neurons and PC12 cells, JNK activity increases soon after NGF deprivation and precedes the increase in c-Jun phosphorylation (Eilers *et al.*, 1998;Ham *et al.*, 1995;Xia *et al.*, 1995). Direct evidence of the c-Jun-activating role of JNK can be seen by the effects of a specific JNK inhibitor, the JIP-1 JBD. JNK interacting protein-1 (JIP-1) is a cytoplasmic scaffold protein that binds both JNK and its upstream activating kinases to facilitate JNK activation (Whitmarsh *et al.*, 1998). When isolated, the JNK-binding domain (JBD) of JIP-1 functions as a decoy substrate to effectively and specifically inhibit JNK (Dickens *et al.*, 1997). Microinjection of an expression vector for this inhibitor into sympathetic neurons has been shown to both prevent c-Jun phosphorylation and promote survival following NGF withdrawal (Eilers *et al.*, 2001). A recent study indicated that c-Jun induction in sympathetic neurons following NGF deprivation is only partially inhibited by mutation of the phosphorylation sites serine 63 and serine 73, although death is significantly delayed, suggesting that c-

Jun phosphorylation at these sites is important but not necessary for neuronal apoptosis (Besirli *et al.*, 2005).

Further elucidation of the signalling pathway that leads to c-Jun activation has involved the identification of components that function upstream of JNK and experiments analysing the effect of expressing various constitutively active expression vectors and dominant negative mutants have proved useful in determining the proteins involved. Most distal to c-Jun and JNK are the Rho-like GTPases Cdc42 and Rac1. Overexpression of activated forms of these proteins in SCG neurons maintained in the presence of NGF activates JNK, elevates levels of c-Jun and also induces apoptosis, whereas dominant negative mutants prevented apoptosis in response to NGF deprivation (Bazenet *et al.*, 1998). The activated mutants of Cdc42 and Rac1 have been found to bind to and modulate the activity of the mixed lineage kinases (MLKs) MLK2 and MLK3, which contain a Cdc42-Rac interacting binding (CRIB) motif in addition to a kinase domain and a leucine zipper region (Bock *et al.*, 2000;Burbelo *et al.*, 1995;Nagata *et al.*, 1998;Teramoto *et al.*, 1996). These MAPK kinase kinases (MAPKKK) appear to function in the signalling pathway that leads to apoptosis in sympathetic neurons since overexpression of MLKs effectively induces apoptosis in the presence of NGF and, conversely, expression of dominant negative forms suppresses death triggered by both NGF deprivation and activated Cdc42 and Rac1 (Xu *et al.*, 2001). There is also evidence that the MAPKKK apoptosis signal-regulating kinase 1 (ASK1) plays an important role in this pathway since constitutively active and dominant negative mutants of this protein have similar effects to the MLK forms above (Kanamoto *et al.*, 2000).

MLKs function to regulate JNK signalling by directly activating the mitogen-activated protein kinase kinases 4 and 7 (MKK4 and MKK7). These dual specificity kinases act directly upstream of JNK to phosphorylate tyrosine and threonine residues within the JNK catalytic domains, which is required for kinase activity (Davis, 2000). Again, evidence from experiments with active and inactive forms of these proteins support their role in this pathway (Xu *et al.*, 2001). Results from experiments performed in cerebellar granule neurons (CGN) undergoing apoptosis due to potassium deprivation indicate that MKK7, rather than MKK4 (also called SEK1) is responsible for activating

the JNK pathway since the latter is not activated in CGN (Trotter *et al.*, 2002). In sympathetic neurons, MKK4 activation by overexpression of an upstream kinase, MEKK1, results in apoptosis in the presence of NGF but expression of a MKK4 dominant negative mutant is not sufficient to protect these cells after NGF deprivation (Eilers *et al.*, 1998), suggesting MKK7 may be the functioning kinase in JNK activation in this model system. Additional components of this Cdc42/Rac1-MLK-MKK-JNK-c-Jun pathway have been identified and include scaffold proteins such as POSH (plenty of SH3s), that aid the formation of multiprotein complexes to link activated Rac1 to the downstream components of the apoptotic cascade (Xu *et al.*, 2003).

The effect of this pathway, particularly its involvement in regulating downstream genes, has been investigated using the inhibitor CEP-1347, a semisynthetic derivative of the metabolite K252a (Wang *et al.*, 2004). Treatment of sympathetic neurons with this small molecule inhibitor blocks NGF deprivation-induced death as well as death following exposure to UV radiation and oxidative stress and saved neurons are able to continue growing, even in the absence of NGF (Maroney *et al.*, 1999). Although CEP-1347 was shown to prevent JNK activation, the exact target of this inhibitor was identified by Maroney and colleagues (2001) who examined the effects of CEP-1347 on upstream JNK regulators in coexpression experiments in COS7 cells. In these experiments, CEP-1347 could not inhibit JNK activation induced by any targets downstream of MLK in the JNK pathway, suggesting that the mixed lineage kinases are the target for inhibition by CEP-1347 (Maroney *et al.*, 2001). This compound functions by competing with ATP for binding to MLK (Wang *et al.*, 2004). CEP-1347 has shown to be effective in preventing neuronal apoptosis in animal models of Parkinson's disease. It was hoped that this might prove a useful treatment in slowing the early progression of disease (Silva *et al.*, 2005;Wang *et al.*, 2004) but phase II/III clinical trials of this compound as a treatment for PD have recently been terminated.

In addition to inhibiting the MLK/JNK pathway, trophic factor-deprived neurons treated with CEP-1347 continue to exert survival activity including neurite outgrowth and neurotransmitter synthesis (Harris *et al.*, 2002). This led to the effects of this inhibitor on survival pathways to be examined. Investigation revealed that CEP-1347 induced activation of Akt and ERK in a MLK-independent manner and blocking either

PI3-K or MEK activity abolishes the survival effects of CEP-1347 (Roux *et al.*, 2002). Studies using the related compound CEP-11004 in NGF-deprived sympathetic neurons indicate that this MLK inhibitor also induces an increase in both *trkA* mRNA and TrkA protein levels. This leads to ligand-independent TrkA receptor activation, resulting in downstream PI3-K activity (Wang *et al.*, 2005). These results suggest that in addition to direct inhibition of the MLK/JNK pathway, indirect activation of the PI3-K pathway, and possibly other survival pathways, occurs when cells are treated with CEP-1347 and related compounds (Figure 1.5). This should be considered when interpreting the effects of this inhibitor.

1.6.4 Mitochondrial pathway

Neurons have the same basic apoptotic machinery as many other cell types, although the role of different components varies depending on the type of neuron and stage of development. *Fas* and *FasL*, both components of the extrinsic apoptotic pathway, show a small increase in expression in sympathetic neurons following trophic factor deprivation, but analysis of *Fas* and *FasL* loss-of-function mutant mice indicates that these genes are not required for NGF-withdrawal induced death (Putcha *et al.*, 2002). This suggests that apoptotic signalling via the extrinsic pathway has little effect on the apoptosis of sympathetic neurons after NGF deprivation. In contrast, the intrinsic mitochondrial pathway plays an essential role in this process. The importance of the BCL-2 family of proteins in sympathetic neurons was first demonstrated by Garcia *et al.* (1992) who showed that overexpression of antiapoptotic BCL-2 rescued these cells from death induced by NGF deprivation. Overexpression of BCL-2 in transgenic mice has since shown that neurons are not only protected from naturally occurring developmental death but also death induced by ischaemic injury or axotomy (Martinou *et al.*, 1994). BCL-2 is widely expressed throughout the developing nervous system although these levels are only maintained in the adult PNS with levels in the CNS being downregulated after birth (Yuan and Yankner, 2000). This is reflected by *bcl-2*$^{-/-}$ null mice, which develop normally but have a subsequent loss of neurons in the PNS after birth, including motor, sensory and sympathetic neurons, indicating that BCL-2 is required for the

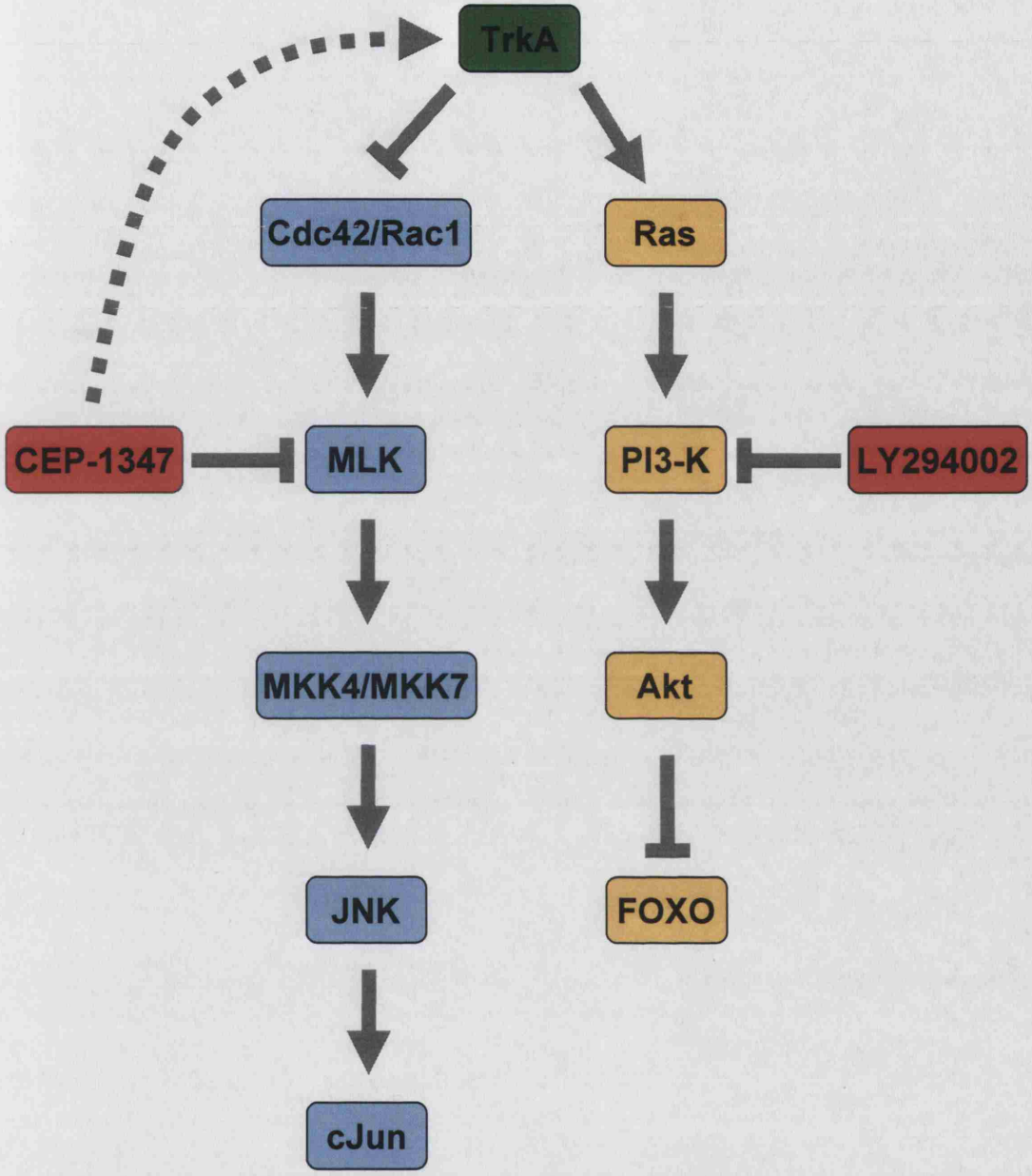


Figure 1.5 Signalling pathways involved in regulating neuronal survival and apoptosis in response to NGF

In the presence of NGF the TrkA receptor is phosphorylated, activating the PI3-K pathway but inhibiting the MLK/JNK pathway. The compound LY294002 competitively inhibits PI3-K and therefore the downstream pathway. CEP-1347 acts to inhibit MLKs and the downstream JNK pathway but appears to indirectly activate the PI3-K pathway via the upregulation of TrkA (Roux *et al.*, 2002; Wang *et al.*, 2005).

survival of cells from specific populations of neurons (Michaelidis *et al.*, 1996). In a similar manner to BCL-2, microinjection of antiapoptotic BCL-X_L protects sympathetic neurons from NGF deprivation-induced death and this protein is also widely expressed in the developing nervous system, although expression continues into adult life (Yuan and Yankner, 2000). In contrast to BCL-2, *bcl-x$_L^{-/-}$* mice die at E13 having extensive apoptosis in the post-mitotic neurons of the brain, spinal cord and dorsal root ganglia (Motoyama *et al.*, 1995). In addition to these antiapoptotic BCL-2 family members, the proapoptotic family member BAX has also been demonstrated to be an essential regulator of neuronal cell death in sympathetic neurons. *Bax$^{-/-}$* mice have an increased number of motor and sympathetic neurons, the latter of which, when cultured *in vitro* in the absence of trophic support, can survive for long periods showing a delay in cytochrome c release (Deckwerth *et al.*, 1996). Conversely, overexpression of BAX through the microinjection of a BAX expression vector into cultured sympathetic neurons induced apoptosis in the presence of NGF, which could be blocked by co-injection of a BCL-X_L expression vector or the baculovirus caspase inhibitor p35 (Vekrellis *et al.*, 1997). These findings suggest a critical role for the interplay of this family of proteins in regulating the apoptosis of sympathetic neurons following neurotrophic factor deprivation.

Similar to apoptosis in other cell types, a redistribution of cyctochrome c from the mitochondria to the cytoplasm is observed in sympathetic neurons during NGF withdrawal-induced apoptosis. This release of cytochome c is blocked by the survival agents CPT-cAMP and cycloheximide and has shown to be necessary for NGF withdrawal-induced apoptosis since death is blocked by the microinjection of an antibody to cytochrome c (Neame *et al.*, 1998). However, in contrast to other non-neuronal cell types, cytochrome c release alone is not sufficient for apoptosis to proceed because microinjection of cytochrome c did not induce apoptosis in NGF-maintained sympathetic neurons (Deshmukh and Johnson, Jr., 1998). This indicates that a second event is required for NGF-deprivation induced apoptosis in sympathetic neurons, which is termed "competence-to-die". Further experiments indicated that this event is independent of both protein synthesis and BAX since microinjection of cytochrome c could induce caspase-dependent death in NGF-deprived cycloheximide-treated and

NGF-deprived BAX-deficient neurons, both of which are normally resistant to apoptosis, and this competence-to-die developed between 15-20 hours after NGF withdrawal (Deshmukh and Johnson, Jr., 1998). Evidence suggests that this second, additional event that is required for apoptosis in neurons may involve the inactivation of the IAP caspase inhibitors. For example, unlike wild type neurons, sympathetic neurons from XIAP deficient mice rapidly undergo apoptosis following the injection of cytochrome c into the cytosol (Potts *et al.*, 2003). Although XIAP resistance to the addition of cytochrome c could be overcome in wild type cells by treatment with exogenous SMAC, an IAP inhibitor, the release of endogenous SMAC was not sufficient to overcome this resistance. In contrast, both *xiap* mRNA and XIAP protein levels are down regulated during the development of competence, which overcomes the resistance to cytosolic cytochrome c and permits caspase activation (Potts *et al.*, 2003). Because neurons are long-lived cells that are not readily replaced, it is likely that this additional step in the neuronal apoptotic pathway allows tighter regulation of cell death, preventing accidental caspase activation due to the unexpected release of cytochrome c.

Other components of the intrinsic apoptotic pathway downstream of the mitochondria have also been show to play an important part in regulating neuronal cell death. In particular, transgenic mice deficient in Apaf-1, caspase-9 or caspase-3 have proved useful in identifying a role for these proteins in neuronal apoptosis as these mice develop a lethal phenotype with a pathology that mostly affects the developing brain. For example, the *Apaf-1*$^{-/-}$ mutant mice exhibit reduced apoptosis in the brain, with enlargement of the periventricular proliferative zone, and these mice die during late embryonic development (Cecconi *et al.*, 1998). *Caspase-9*$^{-/-}$ and *caspase-3*$^{-/-}$ null mutants have a similar phenotype (Kuida *et al.*, 1998;Kuida *et al.*, 1996), indicating the importance of these downstream components in regulating neuronal cell death during nervous system development.

1.7 BH3-only proteins and the regulation of neuronal apoptosis

In a similar manner to other cell types, apoptosis in sympathetic neurons is regulated in part by members of the BH3-only subfamily of the BCL-2 proteins. For example, when overexpressed in sympathetic neurons maintained in the presence of NGF, the BH3-only protein BIM can induce cytochrome c release from the mitochondria and apoptosis (Whitfield *et al.*, 2001). There are several different BIM isoforms formed by alternative splicing, including the variants EL, L and S (extra long, long and short respectively) (O'Connor *et al.*, 1998). BIM_{EL} is the major isoform expressed in sympathetic neurons, cerebellar granule neurons (CGN) and dorsal root ganglia and levels of both mRNA and protein rapidly increase in sympathetic neurons following NGF deprivation (Putcha *et al.*, 2001;Whitfield *et al.*, 2001). The BIM BH3 domain can bind to several BCL-2-like family members with relatively high affinity including BCL-2, BCL-X_L, BCL-W, MCL-1 and A1 (Chen *et al.*, 2005;Kuwana *et al.*, 2005), indicating that BIM may function by inhibiting the antiapoptotic effects of these proteins. There is also *in vitro* evidence that BIM may play a role in BAX activation since BIM can directly bind to and activate BAX (Kuwana *et al.*, 2005). In addition to overexpression studies, experiments using either sympathetic neurons microinjected with *bim* anti-sense oligonucleotides or sympathetic neurons isolated from $bim^{-/-}$ knockout mice indicate that a decrease in BIM levels can partially protect these neurons against apoptosis induced by NGF deprivation (Putcha *et al.*, 2001;Whitfield *et al.*, 2001).

In the nervous system, BIM activity appears to be controlled by a number of mechanisms including transcriptional regulation and post-translational modifications. The increase in BIM levels following NGF deprivation is due, in part, to an increase in transcription from the *bim* promoter. In sympathetic neurons, the activity of the *bim* promoter is negatively regulated by the PI3-K pathway since treatment with the PI3-K inhibitor LY294002 increases *bim* mRNA and protein levels (Gilley *et al.*, 2003). This regulation appears to involve FKHRL1 (FOXO3a), a Forkhead transcription factor of the Forkhead box, class O (FOXO) subfamily that, in the presence of NGF, is phosphorylated by Akt, inhibiting its ability to induce gene expression (reviewed in (Brunet *et al.*, 2001). Following NGF withdrawal, FKHRL1 phosphorylation decreases

allowing it to localise in the nucleus and activate gene transcription. Experiments have demonstrated that overexpression of FKHRL1 can induce *bim* expression and promote BIM-dependent death in sympathetic neurons. In addition, two FOXO-binding sites within the *bim* promoter are required for activation by FKHRL1 as well as activation by NGF withdrawal (Gilley *et al.*, 2003), suggesting that the PI3-K/Akt pathway plays an important part in repressing *bim* transcription in the presence of NGF via the repression of FKHRL1 activity.

In contrast to negative *bim* regulation by the survival PI3-K pathway, the MLK/JNK pathway has also been implicated in BIM regulation. The treatment of sympathetic neurons with the MLK inhibitor CEP-1347 reduces BIM induction following NGF withdrawal, decreasing levels by 85% (Harris and Johnson, Jr., 2001). There is evidence that this regulation by MLK/JNK may occur via c-Jun since the expression of dn-Jun reduces the level of *bim* mRNA induction in NGF-deprived sympathetic neurons by 50%, suggesting a role for c-Jun in the transcriptional regulation of BIM (Sanchez and Yuan, 2001;Whitfield *et al.*, 2001).

In addition to regulating *bim* transcription after NGF withdrawal, the MLK/JNK pathway may also be important in regulating BIM at the post-translational level. In sympathetic neurons NGF withdrawal results in increased phosphorylation of BIM_{EL}, particularly at serine 65, which appears to potentiate the proapoptotic activity of BIM (Putcha *et al.*, 2003). BIM phosphorylation can be blocked by treatment with both the MLK inhibitor CEP-1347 and the JNK inhibitor SP600125, suggesting that both MLK and JNK activity are required for this process (Putcha *et al.*, 2003). In contrast to this, there is evidence from studies in PC12 cells that NGF can promote MEK/MAPK-mediated BIM phosphorylation, which may compromise the proapoptotic activity of BIM and protect neurons from cell death (Biswas and Greene, 2002), indicating that the phosphorylation of BIM at different residues and in different systems may alter the proapoptotic ability of BIM.

In neuronal apoptosis in some situations the BH3-only proteins NOXA and PUMA are known to be transcriptionally regulated, and the latter is transcriptionally induced in sympathetic neurons undergoing cell death after NGF withdrawal. Both the *puma* and *noxa* genes are targets of the tumour suppressor protein p53, a transcription

factor that can regulate several major cell functions, including cell death, through the transactivation of specific target genes (Culmsee and Mattson, 2005). There is some evidence that p53 may be involved in apoptosis in sympathetic neurons since p53 levels increase in response to both NGF deprivation and $p75^{NTR}$ activation and apoptosis due to these stimuli can be blocked by the functional ablation of p53 (Aloyz *et al.*, 1998). The naturally occurring death of sympathetic neurons is inhibited in *p53*$^{-/-}$ mice, supporting the role of p53 in this process (Vogel and Parada, 1998). It is not yet known whether p53 regulates PUMA levels in sympathetic neurons, although it is unlikely that this would be the only target of p53 since *puma*$^{-/-}$ knockout mice are viable with no developmental defects (Jeffers *et al.*, 2003), indicating a degree of redundancy between the BH3-only proteins in neuronal apoptosis.

1.8 DP5

1.8.1 Identification of DP5

The BH3-only protein DP5 (death protein 5) is a polypeptide of 92 amino acids, containing both a BH3 domain and a C-terminal transmembrane region (Imaizumi *et al.*, 1997). It is highly homologous to the human protein Harakiri (HRK), sharing 72% identity at the amino acid level and almost complete conservation of the BH3 domain (Figure 1.6; Imaizumi *et al.*, 1999). DP5 was initially identified by differential display screening for genes upregulated in sympathetic neurons undergoing NGF deprivation-induced apoptosis (Imaizumi *et al.*, 1997). This study demonstrated that *dp5* is normally expressed at low levels but increases dramatically in level following NGF deprivation. Induction does not occur immediately but *dp5* mRNA begins to accumulate by 5 hours after NGF withdrawal, suggesting that *dp5* is induced in response to intracellular events. mRNA levels continue to rise and peak at ~15 hours, concurrent with the commitment point for apoptosis in neurons, at which time cells can no longer be rescued from death by addition of the protein synthesis inhibitor cycloheximide. This *dp5* induction in sympathetic neurons after NGF withdrawal can be blocked by treating the neurons with agents that prevent apoptosis, including cycloheximide, KCl and the cyclic AMP analogue CPTcAMP (Imaizumi *et al.*, 1997). In addition, DP5/HRK is known to be

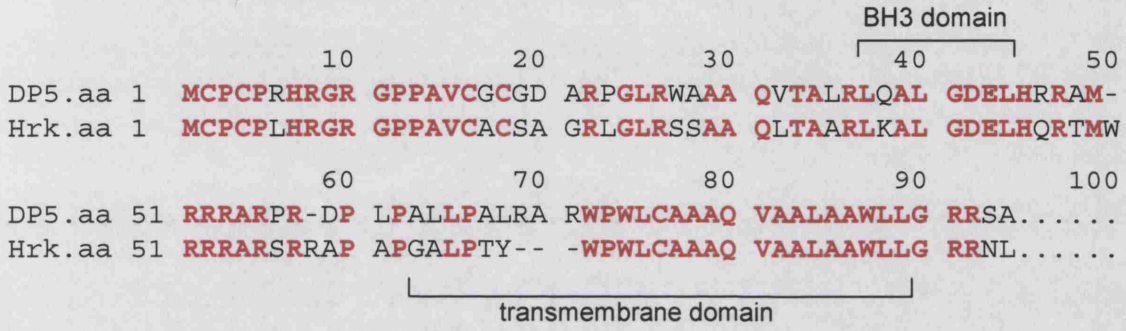


Figure 1.6 Comparison of the rat DP5 and human HRK amino acid sequences

The rat protein DP5 is highly homologous with the human protein HRK, sharing 72% identity at the amino acid level. The BH3 domain and transmembrane domain are indicated and conserved residues are shown in red. (Adapted from Kanazawa *et al.*, 1998)

induced by several different stimuli in a number of neuron types. *dp5* mRNA expression has been shown to increase in PC12 cells following NGF deprivation and in cultured cerebellar granule neurons in response to KCl/serum deprivation (Harris and Johnson, Jr., 2001). Increases in *dp5* mRNA or DP5 protein levels have been observed in rat retinal ganglion cells following optic nerve transection, in rat spinal cords following traumatic injury and in axotomised postnatal mouse motoneurons (Imaizumi *et al.*, 2004;Wakabayashi *et al.*, 2002;Yin *et al.*, 2005). *dp5* mRNA is also induced in cultured rat cortical neurons 6 hours after treatment with β-amyloid protein (Aβ) and appears to be downstream of the changes in cytosolic calcium concentration triggered by Aβ (Imaizumi *et al.*, 1999). In humans, *hrk* expression has been detected in the spinal cord of autopsied patients with ALS compared to non-ALS controls (Shinoe *et al.*, 2001), suggesting a possible role for HRK protein in this neurodegenerative disorder.

Expression of *dp5* mRNA is highly specific to the nervous system of adult rats and examination of developing mice has shown that this gene is primarily expressed in spinal motor neurons and peripheral sensory ganglia of mouse embryos and in the postnatal brain (Imaizumi *et al.*, 1997;Kanazawa *et al.*, 1998). In addition to this, human *hrk* shows high levels of expression in haematopoietic tissues such as the spleen and bone marrow (Sanz *et al.*, 2000). In particular, an increase in *hrk* expression occurs in several growth factor-dependent murine haematopoietic cell lines after growth factor withdrawal or treatment with the chemotherapeutic drug etoposide (Sanz *et al.*, 2000). There is also evidence of a link between hypermethylation of the *hrk* gene and human colorectal and gastric cancers since aberrant methylation near the *hrk* transcriptional start site, a feature thought to play a role in silencing transcription in tumorigenesis, was identified in a number of cancer cell lines. This was associated with a loss of *hrk* expression, which could be restored by methlytransferase inhibition (Obata *et al.*, 2003).

The generation of mutant mice deficient in *dp5* and the analysis of $dp5^{-/-}$ neurons has aided the identification of the functional role of DP5 in neuronal apoptosis (Imaizumi *et al.*, 2004). Whilst $dp5^{-/-}$ mice are viable and show no gross abnormalities during postnatal development, neurons cultured from these mice show some resistance to apoptosis triggered by specific stimuli when compared to neurons from their wild type littermates. Sympathetic neurons from *dp5*-deficient mice demonstrate a delayed onset

of apoptosis following NGF deprivation, with only ~45% of neurons undergoing cell death 36 hours after NGF withdrawal compared to ~65% of dead cells in wild type cultures. This protective effect is transient since most cells are dead by 48 hours in $dp5^{+/+}$, $dp5^{+/-}$ and $dp5^{-/-}$ cultures. In addition to this slower rate of apoptosis, *dp5* deficient sympathetic neurons also have a reduction in mitochondrial membrane potential and a lower level of activated caspase-3 after trophic factor deprivation, implicating these events as important in *dp5* apoptosis signalling. A more pronounced effect of the loss of DP5 is apparent in motoneurons from *dp5* knockout mice (Imaizumi *et al.*, 2004). These cells show a high level of resistance to apoptosis following axotomy, with only a ~10% reduction in neurons 35 days after axotomy compared to a reduction of ~50% in wild type neurons, suggesting that *dp5* expression is important for inducing cell death in motoneurons. Since other BH3-only proteins such as BIM and PUMA are expressed in neurons following these apoptotic insults, it is likely that these may, in part, compensate for a lack of DP5 although DP5 itself does promote cell death to varying extents in different cell types. A similar effect is apparent in $bim^{-/-}$ neurons (Putcha *et al.*, 2001), suggesting a degree of redundancy between the BH3-only proteins. To fully determine the effects of DP5, BIM and PUMA in neuronal apoptosis, it will be useful to analyse neurons from double or triple knockouts or use approaches such as RNA-interference to establish whether eliminating more than one of these proteins can completely abolish cell death in neurons.

1.8.2 Role of DP5 in the intrinsic apoptotic pathway

Overexpression of full length *dp5* by microinjection of a DP5 expression vector into cultured sympathetic neurons is sufficient to induce apoptosis in the presence of NGF, with 70% of injected cells undergoing death with the characteristic morphology of apoptosis by 24 hours. Cells can be rescued from the killing activity of DP5 by co-expression of BCL-2 or BCL-X_L (Imaizumi *et al.*, 1999;Imaizumi *et al.*, 1997). Co-immunoprecipitation studies have show that both DP5 and HRK physically interact with the antiapoptotic proteins BCL-2 and BCL-X_L but deletion of 16 amino acids from the region including the BH3 domain is enough to abolish this interaction and severely

reduce or eliminate killing activity (Inohara *et al.*, 1997). DP5 appears to preferentially interact with certain antiapoptotic BCL-2 family members. Yeast two-hybrid screening identified BCL-X_L and MCL-1 as binding to HRK with a high affinity (Sunayama *et al.*, 2004). Experiments to determine the binding affinity of peptides that include the BH3 domains of different BH3-only proteins to antiapoptotic BCL-2 family members indicated that the HRK BH3 region binds best to BCL-X_L, BCL-W and A1 (Chen *et al.*, 2005). The results from these experiments also suggested that the potency of these peptides for inducing apoptosis corresponded to the range of antiapoptotic proteins that they bound to since the HRK peptide was a less potent killer than the BIM peptide, which was able to bind to a wider range of BCL-2-like proteins.

The killing activity of DP5 depends on the presence of the multidomain proapoptotic protein BAX because overexpression of DP5 does not induce apoptosis in *bax*$^{-/-}$ cerebellar granule neurons (Harris and Johnson, Jr., 2001). Despite this requirement for BAX, DP5 does not physically interact with BAX and induction occurs upstream of the BAX-dependent step as *dp5* is still induced in neurons lacking BAX (Harris and Johnson, Jr., 2001;Inohara *et al.*, 1997). These findings suggest that DP5 may function at least in part by interacting with BCL-X_L and BCL-2 and thereby preventing these antiapoptotic proteins from inhibiting proapoptotic BAX.

Laser scanning confocal microscopy studies investigating the subcellular localisation of FLAG-tagged HRK in 293 cells indicate a pattern of distribution that is consistent with localisation to the membranes of intracellular organelles, similar to BCL-2 and BCL-X_L (Inohara *et al.*, 1997). Further immunological analysis probing HRK in COS cells in the presence of the mitochrondrial marker MitoTracker indicates that HRK predominantly localises to the mitochondria (Sunayama *et al.*, 2004). In addition to physically interacting with various antiapoptotic BCL-2 family members, yeast two-hybrid screening revealed that DP5 interacts with p32, a mitochondrial protein that can form channels as a homotrimer (Sunayama *et al.*, 2004). Various analyses, including *in vitro* binding, co-immunoprecipitation and immunochemical assays verified the specific interaction between these two proteins as well as their co-localisation, both of which require the p32 C-terminal region. Expression of mutants lacking this region, mutants lacking the N-terminal mitochondrial signal sequence or knockdown of p32 by small

interfering RNA protected COS cells against HRK-induced apoptosis (Sunayama *et al.*, 2004), indicating that p32 may play a critical role in the regulation of apoptosis mediated by this BH3-only protein, although how this occurs remains to be determined.

1.8.3 *dp5* regulation

A number of studies have investigated the mechanisms by which DP5 expression is regulated. HRK induction in haematopoietic progenitor cells in response to survival factor deprivation has an expression pattern that correlates with apoptosis induction (Sanz *et al.*, 2000). A downstream regulatory element (DRE) in the 3' untranslated region (3'UTR) of the *hrk* gene was identified and was able to abrogate reporter gene expression if located downstream of the open reading frame (Sanz *et al.*, 2001). This *hrk* silencer sequence binds to the transcriptional repressor DREAM (DRE-antagonist modulator) in interleukin-3 (IL-3)–dependent hematopoietic progenitor cells but, in the absence of IL-3, binding is reduced and the loss of this complex correlates with increased levels of HRK and apoptosis (Sanz *et al.*, 2001). There is some evidence that calcium mobilization and phosphorylation can regulate DREAM transcriptional activity, indicating possible methods by which HRK levels could be controlled in non-neuronal cells (Sanz *et al.*, 2001).

In neurons the MLK/JNK pathway has been implicated in DP5 regulation. The MLK inhibitor CEP-1347 has been shown to completely block JNK activity in hyperpolarised granule neurons and treatment of cells with this compound reduced *dp5* mRNA induction, as analysed by semiquantitative RT-PCR, in both hyperpolarised potassium-deprived cerebellar granule neurons and NGF-deprived sympathetic neurons by ~80% and ~75% respectively (Harris and Johnson, Jr., 2001). This suggests that these levels of *dp5* mRNA induction in sympathetic neurons can be accounted for by the MLK/JNK pathway, although the specificity of CEP-1347 must be considered since this compound can also activate the PI3-K/Akt signalling pathway (Roux *et al.*, 2002;Wang *et al.*, 2005).

There is evidence that the JNK signalling pathway is involved in DP5 regulation in other models of neuronal apoptosis. DP5 induction has been shown to occur rapidly

following spinal cord injury in rats and correlates with an increase in JNK and c-Jun phosphorylation (Yin *et al.*, 2005). DP5 was demonstrated to co-localise with phosphorylated JNK to the same cells within both white and grey matter and reduction of JNK activity by the subcutaneous administration of the small molecule JNK-specific inhibitor SP600125 or a *jnk1* antisense oligonucleotide suppressed both DP5 expression and caspase-3 activation. Inhibition of JNK activity using SP600125 has also been shown to attenuate the upregulation of DP5 in the brain following cerebral ischemia (Gao *et al.*, 2005), suggesting that JNK may regulate *dp5* expression in a range of neuronal cell types in response to a variety of apoptotic stimuli.

1.9 Aims

Although evidence suggests that the proapoptotic BH3-only protein DP5 is regulated transcriptionally and that this involves the activation of the MLK/JNK signalling pathway, little else is known about how *dp5* is regulated in sympathetic neurons, including which are the important regions of the gene and whether any other signalling pathways are involved in controlling gene expression. The aim of my thesis is to further investigate how the *dp5* gene is regulated by NGF withdrawal in sympathetic neurons. This will include trying to locate specific regions of the gene involved in this regulation and determining how regulation occurs. I will also examine the potential pathways involved in controlling *dp5* induction in response to NGF deprivation and will investigate the *dp5* promoter proximal to the transcription initiation site.

To carry out these aims I have made a series of luciferase reporter constructs containing different regions of the *dp5* gene that have been tested by microinjection into sympathetic neurons and transient transfection of PC6.3 cells. Expression of these constructs has then been determined under different conditions such as NGF-deprivation, co-injection or co-transfection with expression vectors and exposure to specific inhibitors. A technique using a dual luciferase assay was established to determine the luciferase activity of different *dp5* reporter genes in response to these different conditions.

If the mechanisms involved in regulating *dp5* expression can be better understood, this will help in furthering our knowledge of how neuronal apoptosis is controlled.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and equipment

Agar Scientific Ltd

No. 5 Dumont forceps, stainless steel

Amersham Biosciences

ECL/ECL plus Western Blotting Detection System; HEPES; Hybond™ ECL™ Nitrocellulose Membrane; Hyperfilm™; Imagemaster Total Lab software; Typhoon™ Phosphorimager 8600; phosphorimager screens; Ultrospec® 2100 Pro spectrophotometer

Becton Dickinson and Co.

Bacto™ agar; Bacto™ tryptone; Bacto™ yeast extract

BDH

Ethidium bromide tablets; ethylenediamine tetra-acetic acid (EDTA); glass slides; HCl; isopropyl alcohol; methanol; $MgCl_2$; NaCl; NaOH; Tris

BioRad Laboratories

Bio-Rad protein assay; gel dryer (model 583); Kaleidoscope prestained standards (molecular weight marker); N,N,N'N'-tetramethylethylenediamine (TEMED); Mini-PROTEAN 3 protein gel and electrophoresis equipment

Carl Zeiss

Axiovert 200 inverted fluorescence microscope with heated stage, incubator and temperature and CO_2 controllers; S100 inverted fluorescence microscope; Axiocam digital camera; Axiovision imaging software

Cedarlane Laboratories Ltd

2.5S nerve growth factor (NGF, for sympathetic neurons)

Cephalon Incorporated

CEP-11004

Citifluor Ltd

Citifluor AF1 glycerol/PBS solution

Clontech

Advantage™-GC 2 PCR kit; rat brain poly-A^+ RNA

Corning Incorporated

Costar 0.22 μm Spin-X centrifuge tube filters

Eppendorf

5810R bench centrifuge; 5415D microcentrifuge; microloader tips; micromanipulator (5171); transjector (5246)

GlobePharm

Foetal calf serum (FCS)

Hayman Ltd

Ethanol

Hettich

Rotina 46R centrifuge

Insight Biotechnology

Isopropyl-β-D-thiogalactopyranoside (IPTG)

Invitrogen

1kb/1kb Plus DNA ladder; agarose, electrophoresis grade; deoxyribonuclease I (DNase I), amplification grade; dithiothreitol (DTT); Dulbecco's phosphate buffered saline

(PBS, -$CaCl_2$, -$MgCl_2$, used with PC6.3 cells); L-15 (Leibovitz, +L-glutamine, +L-amino acids); L-glutamine; Lipofectamine™ 2000 reagent; OptiMEM®I (+GlutaMAX™); One Shot® INV110 chemically competent cells; penicillin/streptomycin; RPMI 1640 Medium (-L-glutamine); subcloning efficiency™ DH5α™ chemically competetent *E. coli*; SOC medium; SuperScript™ II reverse transcriptase; 5X first-strand buffer; 0.1 M DTT

Intracel

Glass capillaries (1.2 mm od x 0.8 mm id x 10 cm)

John Weiss

Dissection scissors and forceps

Kopf Instruments

Gravity puller (model 720)

Lilly

LY294002

Marvel

Non-fat milk powder

National Diagnostics

ProtoGel® 30% (w/v) acrylamide: 0.8% (w/v) bis-acrylamide stock solution (37.5:1); 50x TAE (2.0 M Tris·acetate + 100 mM Na_2EDTA); 10x TBE (0.89 M Tris borate pH 8.3 + 20 mM Na_2EDTA)

New England Biolabs

T4 DNA ligase; T4 DNA ligase buffer

PAA Laboratories

Horse serum

Perkin Elmer Life Sciences Incorporated

[α-^{32}P]dCTP

Promega

2.5S mNGF (for PC6.3 cells); amino acid mixture, complete; deoxyribonucleotide triphosphates (dATP, dCTP, dGTP, dTTP); Dual-Luciferase® Reporter Assay System; GLprimer2; lambda DNA/HindIII DNA ladder; pGEM®-T Easy Vector System I, including T4 DNA ligase and Rapid Ligation buffer; Pfu polymerase; recombinant RNasin® ribonuclease inhibitor; restriction endonucleases; restriction endonuclease digestion buffers; RVprimer3; TNT® T7 Coupled Reticulocyte Lysate System

Qiagen

Plasmid Maxi kit; HighSpeed™ Plasmid Maxi kit; QIAprep Spin Miniprep kit; QIAquick Gel Extraction kit; QIAquick PCR Purification kit; RNeasy® Mini kit

Roche

Klenow polymerase

Saran

Barrier food wrap

Sigma

2-mercaptoethanol; 5-fluoro-2-deoxyuridine; ampicillin; bromophenol blue; diethyl pyrocarbonate (DEPC); dimethyl sulphoxide (DMSO); Dulbecco's Phosphate Buffered Saline (PBS, ±$CaCl_2$, ±$MgCl_2$, used with sympathetic neurons); Dulbecco's Modified Eagle's Medium (DMEM); ethylene glycol-bis (β-aminoethyl ether)-N,N,N'N'-tetraacetic acid (EGTA); fetal bovine serum (FCS, used with sympathetic neurons); glycerol; guinea pig IgG; Hoechst 33342; laminin; paraformaldehyde; poly-L-lysine; Ponceau S solution (0.1% (w/v) in 5% acetic acid (v/v)); protease inhibitor (PI) cocktail; REDTaq™ DNA polymerase; REDTaq™ DNA polymerase buffer; sodium acetate; sodium dodecyl sulphate (SDS); Triton X-100; trypsin; 1x trypsin/EDTA; Tween-20; uridine

Sigma-Genosys Ltd

PCR primers/oligonucleotides; T7 primer; SP6 primer

Sorvall

RC5B Plus Superspeed refrigerated centrifuge; Discovery™100 ultracentrifuge; ultracentrifuge tubes and lids;

Stratagene

QuikChange® II XL Site-Directed Mutagenesis kit including XL-Gold® ultracompetent cells and DpnI restriction enzyme

Upstate Biotechnology

Type I collagen, rat tail

VWR International

13 mm coverslips

Whatman Scientific

3MM paper

Worthington Biochemical Corporation

Type 2 collagenase

2.1.2 Antibodies

Chemicon International

Mouse anti-nerve growth factor monoclonal antibody, clone 27/21

Jackson Immunoresearch Laboratories

Donkey anti-goat FITC-conjugated IgG

Donkey anti-guinea pig rhodamine-conjugated IgG

Promega

Goat anti-luciferase polyclonal antibody

Santa Cruz

Goat anti-E4BP4 polyclonal antibody (V-19)

Donkey anti-goat horse radish peroxidase-conjugated Ig

2.1.3 Plasmids

pGL3-basic, pGL3-P, pRLT-K and pGEM T-Easy were purchased from Promgea

The *c-jun* luciferase (*c-jun*-LUC) reporter was generated by Dr. Jonathan Gilley by inserting the human *c-jun* promoter (-1,600 to +170) into pGL3-basic. The j1j2 mutant had mutations at the TPA response elements jun1 (-70) and jun2 (-190) in the *c-jun* promoter as described by Angel *et al.* (1988).

wtMLK3 and kdMLK3 expression constructs were kindly provided by Dr. C.E. Bazenet and were described by Tibbles *et al.* (1996) and Kiefer *et al.* (1996).

pcDNA3.1 and the pcDNA3.1 E4BP4 expression vector were kindly provided by Dr. Jenny Yeung.

2.1.4 Primers and oligonucleotides

Sequencing primers

T7 5'-TAATACGACTCACTATAGGG-3'

SP6 5'-GATTTAGGTGACACTATAG-3'

GL2 5'-CTTTATGTTTTTGGCGTCTTCCA-3'

RV3 5'-CTAGCAAAATAGGCTGTCCC-3'

dp5 3'UTR specific	5'-ATACTGACTGAAGCCTAGTG-3'
	5'-GTGGACTGTTAGAACACAAG-3'
	5'-CGGAACGACCGTCTAACGAT-3'
	5'-GTAACGATCAGATTCCCGT-3'
	5'-TTTGGGGAGCAGGGGAAATCG-3'
	5'-TTCAGAAGTTTCTAGAAGGG-3'
	5'-TTAAAATGGAGAGAGGAGCC-3'
	5'-TATATTTCTCTGAATAACCC-3'
	5'-AGTATTTACAGAGGACTTGGC-3'
	5'-AATCTGGGCCTTGGCTCGC-3'
	5'-TCACTATTGGGGACGACGGC-3'
	5'-AGACTTGTGTTCTAACAGTCC-3'
	5'-AAGCCTTGCTGGCAGATTGC-3'
	5'-TAGGATGGGGCTCTCTCAGGG-3'
	5'-TGTAAGACTCTGTCTGTACC-3'
	5'-GGAGAGGCAGTAACTTGGG-3'
	5'-AAGGAGCTGGTTGTGTGAGG-3'
	5'-AGCCAGCAGCTTTTAGACC-3'

RT-PCR primers

nfm	5'-ACGCTGGACTCGCTGGGCAA-3'	(forward)
	5'-GCGAGCGCGCTGCGCTTGTA-3'	(reverse)
dp5	5'-AGACCCAGCCCGGACCGAGCAA-3'	(forward)
	5'-ATAGCACTGAGGTGGCTATC-3'	(reverse)
e4bp4	5'-GCTCTCGGATGTGTCTGAG-3'	(forward)
	5'-GGGGACCTGCTGCTCGTCT-3'	(reverse)

EMSA oligonucleotides

wild type E4BP4	5'-CTAGGCCGGATGATGTAACCCCCT-3'	(forward)
	5'-CTAGAGGGGGTTACATCATCCGGC-3'	(reverse)
mutant E4BP4	5'-CTAGGCCGGATAACGTCTCCCCCT-3'	(forward)
	5'-CTAGAGGGGGAGACGTTATCCGGC-3'	(reverse)

2.1.5 Stock solutions

Solutions were made up using MilliQ deionised water unless otherwise specified.

TE	10 mM Tris-Cl pH 8.0 1 mM EDTA
6 x DNA loading buffer	0.24% bromophenol blue 0.24% xylene cyanol 30% glycerol 60 mM EDTA, pH 8.0
LB	1% Bacto™ tryptone 0.5% Bacto™ yeast extract 17 mM NaCl
LB agar	LB containing 1.5% Bacto™ agar
NaCl-saturated isopropanol:water	50% isopropanol 50% sterile water NaCl added until 2 phases appear
DEPC-treated water	0.1% diethyl pyrocarbonate
2x SDS sample buffer	1x Tris-Cl, pH6.8 20% glycerol 4% SDS

	0.2 M DTT
	0.001% bromophenol blue
SDS polyacrylamide gels	<u>stacking gel</u>
	4% acrylamide
	0.375 M Tris-Cl, pH 6.8
	0.1% SDS
	0.05% APS
	0.1% TEMED
	<u>10% resolving gel</u>
	10% acrylamide
	0.375 M Tris-Cl, pH 8.8
	0.1% SDS
	0.05% APS
	0.1 % TEMED
5x Running Buffer	25 mM Tris
	192 mM glycine
	0.1% SDS
Transfer Buffer	25 mM Tris, pH 8.0
	0.2 M glycine
	20% methanol
Tris Buffered Saline-Tween (TBS-T)	10 mM Tris, pH 8.0
	150 mM NaCl
	0.05% Tween-20
5% milk/TBS-T	5% (w/v) milk powder
	TBS-T as above
2x Dialysis Buffer	40 mM Hepes
	2 mM $MgCl_2$

	1 mM EDTA
	50 mM KCl
	20% glycerol
(add immediately prior to use)	1 mM DTT
	1 mM PI cocktail
TM Buffer	100 mM Tris, pH 8.0
	50 mM $MgCl_2$
6% EMSA gel	6% acrylamide
	0.5x TBE
	15% APS
	5% TEMED
4x E4 Binding Buffer	25 mM Hepes pH 8.0
	1 mM EDTA
	10% glycerol
	50 mM KCl
(add immediately prior to use)	2 mM DTT
	2% PI cocktail

2.2 Methods

2.2.1 DNA manipulations

2.2.1.1 Plasmid construction

Cloning of the rat dp5 gene

The rat *dp5* promoter was cloned and sequenced by Dr. Jonathan Gilley. To obtain the sequence upstream of the initiator codon, 5' rapid amplification of cDNA ends (5'RACE) was performed on rat brain mRNA using the Marathon™ cDNA amplification kit (Clontech) with the *dp5*-specific primer 5'-CTGCAGCCGCAGCGCGGTCACC-3'. To isolate the *dp5* promoter, the 5' RACE product was used as a probe to screen the rat P1 artifical chromosome (PAC) library RPCI31 (generated by P.Y. Woon and P. de Jong, UK Human Genome Mapping Project Resource Centre, Cambridge, UK). A 9 kb fragment containing the 5' end of the promoter was obtained but could not be cloned. Therefore 4 kb from the 5' end of this fragment containing the sequence from upstream of exon 1 was cloned. A primer from the 3' end of the 4 kb fragment was used with a primer from exon 1 to generate the missing 0.5 kb of promoter sequence between the start codon and upstream 4 kb fragment by PCR. These sequences were ligated at an EcoRI restriction site to generate a 4.5 kb fragment in pGEM®-T Easy, which was then subcloned into pGL3-basic using BglII and NcoI restriction enzyme digestion sites.

dp5 promoter reporter constructs

dp5-LUC reporter constructs containing between 150 bp and 4.5 kb of the *dp5* promoter were generated by Dr. Jonathan Gilley by subcloning different lengths of the sequence directly 5' to the *dp5* initiator codon into pGL3-basic.

Addtional *dp5*-LUC constructs were generated that contain small regions of the *dp5* promoter each stopping upstream of potential transcription factor binding sites and all immediately 5' of the initiator codon. The promoter fragments were generated using the same reverse primer that corresponded to a sequence from 37 bases downstream of the transcriptional start site incorporating a BglII restriction site 5'-CACAGATCTGGC

GTGCGGAGGAGCCTCCC-3' and the following forward primers, all incorporating a SpeI restriction site: Δ-66 fragment 5'-ATAACTAGTCCGCCCCCTCCACCATGTGA-3', Δ-51 fragment 5'-ATAACTAGTTGTGACACTTTAATTAATAA-3', Δ-43 fragment 5'-ATAACT AGTTTTAATTAATAATAGCGCCG-3' and Δ-25 fragment 5'-ATAACTAGTGCGCC GTCAGCACAACAAAC-3'. These promoter fragments were cloned directly into pGL3-basic that had been digested with NheI and BglII and fragment integrity was confirmed by DNA sequencing.

dp5 reporter constructs containing regions from the intron and the 3' untranslated region (3'UTR)

To obtain luciferase reporter constructs containing part of the *dp5* intron, a 395 bp region of the intron that is highly conserved in sequence between the rat, mouse and human *dp5* genes (fragment I) was generated by PCR using the forward primer 5'-ATAGGATCCACTAGACTACCTTCCGAGAGTGG-3' and reverse primer 5'-ATAGG ATCCACCCTCCAAAATATTGGTCTCACC-3', both of which incorporate a BamHI restriction site. Fragment I was cloned directly into pGEM®-T Easy and the sequence integrity was determined by DNA sequencing. The intron fragment was subcloned into 1kb*dp5*-LUC at a BamHI site downstream of the SV40 polyadenylation (polyA) termination sequence, and the fragment orientation was determined by restriction digestion with EcoRV and XbaI. A construct containing fragment I in the 5' to 3' (forward) orientation was obtained and named 1kb*dp5*-LUC+I(F).

A reporter construct containing the *dp5* conserved intron fragment upstream of the SV40 promoter was made by inserting the intron fragment (I), digested from 1kb*dp5*-LUC+I(F) using BamHI, into pGL3-P at a compatible BglII restriction site. To ensure that fragment I was inserted in the 5'-3' (forward) orientation, the construct, named SV40-LUC+I(F), was digested with EcoRV and SalI.

dp5-LUC reporter constructs containing different regions of the *dp5* 3'UTR were generated as follows. A 645 bp fragment containing the *dp5* stop codon and the 5' end of the *dp5* 3'UTR (fragment A) was generated by PCR using a forward primer incorporating a 5' SpeI restriction site 5'-ATAACTAGTCGGCCTGGCTGCT CGGCAGGC-3' and a reverse primer 5'-TGAGATCTAGAGAGCTGACTC-3'. This

fragment was cloned directly into pGEM®-T Easy and fragment integrity was determined by DNA sequencing. Fragment A was then subcloned into an XbaI site downstream of luciferase and upstream of the SV40 transcription termination signal in 1kb*dp5*-LUC, a construct containing 1 kb of the *dp5* promoter, and the orientation was confirmed by restriction digestion with NcoI and XbaI. Constructs containing fragment A in both 5' to 3' (forward) and 3' to 5' (reverse) orientations were obtained and named 1kb*dp5*-LUC+A(F) and 1kb*dp5*-LUC+A(R) respectively. The remaining sequence of the *dp5* 3'UTR was generated by PCR in two stages: a 2.36 kb fragment (fragment B) was generated by using a forward primer incorporating a 5' SpeI restriction site 5'-CTCACTAGTTC TCATGGCGGTCATAGC-3' and a reverse primer downstream of a *NarI* restriction site 5'-GAGCAGCCAGCAGCTTTTAG-3', and a 2.02 kb fragment (fragment C) was made using a forward primer upstream of a NarI restriction site 5'-TCTG AGATCATTGCTAAGGAGC-3' and a reverse primer incorporating a 3' SpeI restriction site 5'-ATAACTAGTGTACAAAGCATCCAGGCAGGGAGC-3'. Fragments B and C were cloned directly into pGEM®-T Easy and fragment integrity was determined by sequencing. To construct 1kb*dp5*-LUC+3'UTR, both fragments B and C were excised using SpeI and NarI and inserted together into 1kb*dp5*-LUC+A(F) at an XbaI site downstream of fragment A. Fragment orientation and positioning was confirmed by restriction enzyme analysis and DNA sequencing.

1kb*dp5*-LUC+A1 was made by generating a 109 bp fragment (fragment A1), containing the *dp5* 3'UTR from exon 1, by PCR using the fragment A forward primer and a reverse primer incorporating a SpeI restriction site 5'-ATAACTAGTTTTCGCCAACCTGTTGCTCGGTCC-3'. Fragment A1 was cloned into pGEM®-T Easy and fragment integrity was determined by DNA sequencing before the DNA fragment was subcloned into the XbaI site downstream of luciferase in 1kb*dp5*-LUC. The first 536 bp of the 3'UTR in exon 2 (fragment A2) was cloned by Dr. Jonathan Gilley who generated a construct containing this region as well as 1 kb of the *dp5* promoter (1kb*dp5*-LUC+A2) by subcloning fragment A2 into an XbaI site downstream of luciferase in 1kb*dp5*-LUC.

To generate a construct containing 1kb of the *dp5* promoter, the conserved region of the intron and the 3'UTR (named 1kb*dp5*-LUC+ALL), fragment I was excised from

the construct 1kb*dp5*-LUC+I(F) using BamHI and inserted into partially digested 1kb*dp5*-LUC+3'UTR downstream of the poly-A termination signal. Insertion into the appropriate site and the orientation of the fragment were checked by digestion with EcoRV and SalI restriction endonucleases.

2.2.1.2 Polymerase chain reaction (PCR)

The different regions of the *dp5* gene were cloned from a PAC library or, in the case of fragment A, rat brain cDNA, using the primers described above (Section 2.1.4). In most cases DNA was amplified using the Advantage™-GC 2 PCR kit for GC-rich sequences and a typical 25 µl of reaction was prepared using 1x GC 2 PCR Buffer, 1 M GC Melt, 1 µl of PAC template or 2.5 µl of cDNA template, 0.1 µg of each primer, 0.2 mM of each dNTP and 1x Advantage-GC 2 polymerase mix. The intron fragment was amplified using Pfu DNA polymerase. A 50 µl reaction was prepared using 1x reaction buffer with $MgSO_4$, 2 µl of PAC template, 0.1 µg of each primer and 0.2 mM of each dNTP. Typical reaction conditions were an initial denaturation at 94°C for 2 minutes, then 35-40 cycles of 94°C for 30 seconds, *x*°C for 30 seconds (see Table 2.1) and 72°C for 1 minute per kilobase of expected product, and then a final extension of 72°C for 5 minutes.

Table 2.1 Annealing temperature of different primers used for cloning different regions of the *dp5* gene

Primers	Annealing Temperature
Δ-51, Δ-43	55°C
Δ-66, Δ-25	65°C
Intron Fragment (I),	60°C
3'UTR Fragment A, Fragment C, Fragment A1	57°C
3'UTR Fragment B	64°C

PCR products were analysed by agarose gel electrophoresis. In the case of the *dp5* promoter construct deletions, due to the small size of each product and to avoid the loss of any DNA, the PCR products were purified using a QIAquick PCR Purification kit following the manufacturer's protocol and eluted into 30 µl of the elution buffer provided. The products were digested with SpeI and BglII before being purified again, using the same method, and were directly ligated into digested pGL3-basic.

2.2.1.3 Restriction endonuclease digestion

Typically, an excess of restriction enzyme (10 units) was used to digest ~0.5 µg of plasmid DNA in a 30 µl reaction with the appropriate 1x restriction enzyme buffer. Reactions were left for 1-2 hours at 37°C and the product was analysed by agarose gel electrophoresis (see Section 2.2.1.4). For partial digestion, 1 µg of plasmid DNA was digested with 5 units of restriction enzyme with 1x restriction enzyme buffer in a 50 µl reaction. The reaction was left at 37°C and after 20, 25 and 30 minutes 10 µl was removed and the reaction stopped by the addition of 1x gel loading buffer. Samples were analysed by agarose gel electrophoresis to obtain the appropriately digested construct.

2.2.1.4 Agarose gel electrophoresis of DNA

After PCR or restriction endonuclease digestion, DNA was analysed by agarose gel electrophoresis. 0.8-1.5% agarose gels were prepared using 1x TAE or 1x TBE buffer containing 0.5 µg/ml of ethidium bromide. 1x gel loading buffer (final concentration) was added to the samples before they were run on the gel, along with 1 µg of 1 kb or 1 kb plus DNA ladder or Lambda DNA/HindIII markers, at 80-120 V. After electrophoresis, the DNA was visualised using a Uvi tec UV transilluminator and the size determined by comparison with the DNA ladder. DNA was purified using a QIAquick Gel Extraction kit following the protocol provided and was eluted into 30 µl of elution buffer.

2.2.1.5 Phosphatase treatment and ligation of DNA

Before being used in a ligation reaction, digested vector DNA was phosphatase treated to minimise self ligation of the vector DNA molecules. Treatment was generally carried out directly after endonuclease digestion, using 1 unit of calf intestine alkaline phosphatase (CIP) and 1x CIP buffer. Reactions were left at 37°C for 5 minutes after which time the reaction was stopped by the addition of 1x gel loading buffer and the sample analysed by agarose gel electrophoresis. After partial digestion, plasmid DNA was phosphatase treated after gel electrophorsis and gel extraction using 2 units of shrimp alkaline phosphatase (SAP) and 1x SAP buffer at 37°C for 10 minutes. This was then deactivated by heating at 65°C for 15 minutes.

Digested plasmid and insert DNA molecules were ligated by adding 400 units of T4 DNA ligase (NEB) and 1x T4 DNA ligase buffer to vector and insert DNA (mixed at a ratio of approximately 2:1 or 3:1 insert to vector molecules). Ligation reactions were either left for 2-3 hours at room temperature or overnight at 16°C. PCR products were cloned by mixing 3 µl of purified PCR product with 50 ng of pGEM® -T Easy vector and 3 Weiss units of T4 DNA ligase (Promega) with 1x Rapid Ligation buffer. Reactions were left at room temperature for 1-2 hours.

2.2.1.6 Bacterial transformation

Subcloning efficiency™ DH5α™ chemically competent *E. coli* was used for bacterial transformation. Ligated DNA (10 µl reaction) or plasmids (~1 µg) were mixed with 100 µl of DH5α cells and incubated on ice for 30 minutes. This was followed by a heat-shock at 37°C for 45 seconds and then a further incubation on ice for 2 minutes. 800 µl of LB medium was added and the cells were incubated at 37°C for 1 hour. After this time, 100 µl of cells were spread onto LB-agar plates containing either ampicillin alone at 50 µg/ml or, for the transformation of pGEM®-T Easy plasmids, ampicillin at 50 µg/ml, 25 µg/ml of X-gal and 0.5 mM IPTG. The remainder of the cells were pelleted by spinning in a microfuge at 16,100 x g for 1 minute. These cells were resuspended in

100 µl of LB medium and were spread onto the appropriate plates. All plates were incubated at 37°C overnight.

To transform the 3'UTR fragment A PCR product, a $dam^{-/-}$ *E. coli* strain was used (One Shot® INV110 chemically competent cells). 5 µl of ligated fragment A was added to the INV110 competent cells and then incubated on ice for 30 minutes. The cells were heat-shocked at 42°C for 30 seconds and 250 µl of pre-warmed SOC medium was added. The cells were shaken at 225 rpm at 37°C for 1 hour and then spread (as above) on LB-agar plates containing ampicillin (50 µg/ml), tetracycline (10 µg/µl), X-gal (25 µg/ml) and IPTG (0.5 mM). The plates were incubated overnight at 37°C.

2.2.1.7 Plasmid preparation

Small scale plasmid preparation (minipreps)

Single transformed bacterial colonies were picked and added to 5 ml of LB medium containing 50 µg/ml of ampicillin. In the case of pGEM® -T Easy plasmids and INV110 transformed cells blue/white selection was carried out and only white colonies were picked. These were shaken at 250-300 rpm and left growing overnight at 37°C. 4 ml of the liquid culture was then spun at 5000 x g for 5 minutes at 4°C and the plasmid DNA was extracted from the cells using a QIAprep Spin Miniprep kit following the manufacturer's guidelines for using a microcentrifuge. This procedure involved the alkaline lysis of bacterial cells followed by neutralisation and clearance of the lysate by centrifugation and the adsorption and purification of DNA onto a silica-gel membrane in the presence of high salt. Salts were then removed by washing the membrane and DNA was eluted in 50 µl of the low-salt elution buffer provided.

The remaining 1 ml of liquid culture was used to make a glycerol stock by mixing equal volumes of bacterial culture and sterile 50% glycerol. In some cases large scale cultures were inoculated directly.

Large scale plasmid preparation (maxipreps)

5 ml of LB medium containing ampicillin at 50 μg/ml was inoculated with 5-10 μl of a plasmid glycerol stock or small scale culture and was shaken at 250-300 rpm for 6-10 hours at 37°C. This was used to inoculate 200 ml of LB medium containing ampicillin (50 μg/ml) and was shaken at 250 rpm overnight at 37°C. The bacterial cells were harvested by centrifugation at 6000 x g for 15 minutes at 4°C and plasmid DNA was purified using a Qiagen Plasmid Maxi kit or a HighSpeed™ Plasmid Maxi kit, following the manufacturer's instructions. Both of these methods are based on the alkaline lysis of bacterial cells followed by the binding of plasmid DNA to an anion-exchange resin under conditions of low-salt and the appropriate pH. Impurities were removed by a medium-salt wash and plasmid DNA was then eluted in a high-salt buffer before being concentrated and desalted by isopropanol precipitation. The DNA concentration was determined by measuring the optical density at 260 nm and plasmid integrity was confirmed by restriction enzyme analysis.

Caesium chloride gradient purification

In the case of plasmid DNA purified using Qiagen Plasmid Maxi kits, further purification using a caesium chloride gradient was performed. The DNA sample was mixed with 11 ml of CsCl/TE (10 g/ 9 ml) and 400 μl of ethidium bromide (10 mg/ml) and was transferred to an ultracentrifuge tube using a needle and syringe. Tubes were balanced to within 0.01 g and were sealed with a plastic stopper and metal cap. Samples were spun at 57,000 rpm for at least 18 hours at 20°C using a Sorvall Discovery 90 ultracentrifuge, with slow deceleration. The lower ethidium bromide-stained band contained supercoiled DNA and was extracted by first piercing the tube at the top with a 21 G needle to release the air and then inserting an 18 G needle and syringe below the band to extract it. The DNA was transferred to a 15 ml centrifuge tube and was extracted from the ethidium bromide by adding 1 ml of NaCl-saturated isopropanol. The DNA was mixed thoroughly and the two phases were allowed to separate, after which the top phase was removed. This stage was repeated until both phases were completely colourless.

To precipitate the DNA, the colourless lower phase containing the DNA was transferred to a 50 ml centrifuge tube with 3 volumes of water and then 2 volumes (total volume) of ethanol were added. This mixture was incubated on ice for 10 minutes and was then spun at 5000 x g for 1 hour at 4°C. The supernatant was removed and 20 ml of 70% ethanol was added before the tubes were spun again at 5000 x g for 30 minutes at 4°C. All of the supernatant was removed before a final spin at 5000 x g for 5 minutes at 4°C. Any remaining traces of supernatant were removed and the pellet was air dried before the DNA was redissolved in a suitable volume of TE.

2.2.1.8 DNA sequencing

DNA sequencing was performed at the Wolfson Institute for Biomedical Research (WIBR) Scientific Services Unit using a Beckman Coulter CEQ 8000 genetic analysis system. Plasmid DNA was diluted to 9-16 fmoles/µl and was sequenced using either T7 or SP6 primers (provided by WIBR) or the luciferase primer GL2. The sequence was analysed using Chromas2 and MacVector software. Alternatively, sequencing was carried out using the ABI PRISM® BigDye™ Terminator Cycle Sequencing Ready Reaction kit. 15 µl reactions were prepared using 3.5 µl of miniprep DNA with 6 µl of BigDye Reaction Ready mix and 50 pmoles of primer (T7, SP6, GL2, RV3 or *dp5* sequence-specific primer). The PCR reactions were performed at 96°C for 30 seconds, 50°C for 30 seconds and 60°C for 4 minutes for 25 cycles. 2 µl of 3 M sodium acetate was added and the sample mixed before this was added to 50 µl of absolute ethanol. The reactions were precipitated on ice for a minimum of 15 minutes and then spun at 16,100 x g for 15-30 minutes at 4°C. The supernatant was removed and samples were washed in 100 µl of 70% ethanol. Reactions were left at room temperature for 5 minutes and then spun at 16,000 x g for 10 minutes at room temperature. The supernatant was removed, the wash step repeated and the DNA pellet left to air dry. Samples were run on an ABI Prism 377 DNA sequencer by Mr. Martin Woodward or Ms. Danielle Fletcher.

2.2.1.9 Site-directed mutagenesis

Site-directed mutagenesis was carried out using the QuikChange® II XL Site-Directed Mutagenesis kit to introduce three point mutations into the E4BP4-binding site in reporter constructs containing both 1 kb and 150 bp of the *dp5* promoter (named 1kb*dp5*-LUC+mut and 150bp*dp5*-LUC+mut respectively). Mutagenesis was carried out following the manufacturer's guidelines using primers that both incorporated the desired mutation and corresponded to the sequence on each strand.

5'-CCCGGGCCGG**AT**A**A**CGT**CT**CCCCCTCCCCGCG-3'

3'-GGGCCCGGCC**TA**T**T**GCA**GA**GGGGGAGGGGCGC-5'

50 µl reactions were prepared with 1x reaction buffer, 10 ng of double-stranded DNA plasmid template, 125 ng of each primer, 1 µl of dNTP mix and 3 µl of QuikSolution and thermal cycling carried out with an initial denaturation at 95°C for 1 minute followed by 18 cycles of 95°C for 50 seconds, 60°C for 60 seconds and 68°C for 12 minutes, and a final extension of 68°C for 7 minutes. The reactions were then incubated with 10 units of Dpn1 restriction enzyme at 37°C for 1 hour to digest the parental supercoiled plasmid DNA. Mutated DNA was transformed into XL-Gold® Ultracompetent cells following the protocol provided. 250 µl of transformed cells were spread onto LB-agar plates containing ampicillin (50 µg/ml), X-gal (80 µg/ml) and IPTG (20 mM) and were incubated at 37°C for 20 hours. After DNA amplification, sequence integrity and the incorporation of the correct point mutations was determined by DNA sequencing.

To generate a construct containing 1 kb of the *dp5* promoter as well as the *dp5* intron and 3'UTR, the promoter sequence containing the point mutations was excised from 1kb*dp5*-LUC+mut by restriction digestion with EcoRI and NcoI, and was subcloned into digested 1kb*dp5*-LUC+ALL. Incorporation of the mutated fragment was confirmed by DNA sequencing.

2.2.2 Tissue culture

2.2.2.1 Isolation and culture of sympathetic neurons

Sympathetic neurons were prepared from the superior cervical ganglia (SCG) of 1-day-old Sprague Dawley rats (supplied by the Biological Services Unit, University College London). Neurons were isolated and cultured as described (Whitfield, Neame and Ham, 2004). After dissection, the SCG were desheathed in pre-warmed L15 (Leibovitz) medium containing 0.1% foetal calf serum (FCS) plus penicillin/streptomycin. Ganglia were dissociated by treatment with 0.025% trypsin (in PBS lacking Mg^{2+} and Ca^{2+}) and 0.2% collagenase type 2 (in PBS containing Mg^{2+} and Ca^{2+}), each for thirty minutes at 37°C, followed by gentle trituration through the tip of a Gilson P1000 pipette. Cells were collected by centrifugation at 200 x g for 10 minutes at room temperature and resuspended in 10 ml of Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FCS, 2 mM glutamine and penicillin/streptomycin. Unless stated otherwise, SCG growth medium was also supplemented immediately prior to use with 2.5S NGF at 50 ng/ml and the anti-mitotic agents fluorodeoxyuridine and uridine at a final concentration of 20 μM to limit the proliferation of non-neuronal cells. To remove non-neuronal cells, the cell suspension was preplated in an uncoated 10 cm tissue culture dish for 2-3 hours. After this time, the SCG neurons in the supernatant were collected by centrifugation at 200 x g for 10 minutes at room temperature and the cells resuspended in SCG growth medium. The cells were then plated onto 13 mm diameter glass coverslips coated with poly-L-lysine and laminin at a density of 6400-8000 neurons per coverslip. Coverslips were placed in 3.5 cm dishes and after a two hour incubation at 37°C in 10% CO_2, the dishes were flooded with 2 ml of SCG growth medium and maintained in these conditions for 5-7 days before being used in experiments. Neurons were refed with fresh medium after 1 and 2 days *in vitro* and then every 2-3 days.

In NGF withdrawal experiments, the medium was carefully removed from each dish and the cells were very gently rinsed twice with prewarmed SCG growth medium lacking NGF and anti-mitotic agents. The neurons were then refed with SCG growth medium supplemented with neutralising anti-NGF antibody at 100 ng/ml or with fresh medium containing NGF. In experiments involving the treatment of sympathetic

neurons with chemical compounds, cells were refed with medium containing the appropriate concentration of the compound being used (see results chapters) or the equivalent volume of DMSO as a control.

2.2.2.2 PC6.3 cell culture

Tissue culture plastic used for growing the PC6.3 subline of the PC12 cell line (Pittman *et al*, 1993) was treated with rat tail type 1 collagen at 10 mg/ml in PBS for at least 1 hour before use, and then rinsed twice with PBS. Naïve PC6.3 cells were cultured in RPMI 1640 medium containing 10% horse serum, 5% FCS, 2 mM glutamine and penicillin/streptomycin (naïve PC6.3 growth medium). These cells were subcultured every 7 days by rinsing the cells with PBS and then incubating with 1 x trypsin/EDTA for 5 minutes. The cells were harvested by centrifugation at 200 x g for 5 minutes and the pellet was resuspended and plated in naïve PC6.3 growth medium. To obtain a differentiated neuronal phenotype, the cells were plated at a density of $1x10^6$ cells per 10 cm dish in RPMI 1640 medium supplemented with 2% horse serum, 1% FCS, 2 mM glutamine, penicillin/streptomycin and NGF at 100 ng/ml. All PC6.3 cells were maintained at 37°C in 5% CO_2 and the medium was changed every 2-3 days.

2.2.3 Microinjection

2.2.3.1 Microinjection of sympathetic neurons

Sympathetic neurons were cultured as described above (Section 2.2.2.1) and microinjected after 5-7 days *in vitro* with different DNA reporter constructs and expression vectors according to individual experiments at the concentrations indicated (see results). The injection mix was prepared immediately prior to microinjection and contained the DNA to be tested in 0.5x PBS ($-Ca^{2+}$, $-Mg^{2+}$). Neurons to be analysed by immunofluorescence were co-injected with purified guinea pig IgG at 2.5 mg/ml to act as a marker for injected cells. For cells to be analysed by the dual Luciferase assay, 0.005 mg/ml of the *Renilla* construct pRL-TK was co-injected, controlling for variations

between coverslips. Injection mixes were spun through a 0.22 μm filter Spin-X tube for 30 minutes at 4°C before microinjection to remove any particulate matter.

Microinjection needles were pulled from glass capillary tubes using a Kopf Instruments gravity puller (model 720) and were either loaded using Eppendorf microloaders or backloaded using capillary action. Microinjection was performed using a Zeiss Axiovert 200 microscope with an Eppendorf transjector (model 5246) and micromanipulator (model 5171). During injection the dish containing cells was placed on a temperature-regulated stage in a chamber with controlled CO_2 levels. DNA was injected directly into the nucleus and either 200 neurons for immunofluorescence or 150 neurons for luciferase assay were injected per coverslip. After microinjection, cells were treated as described for individual experiments. More than 50% of neurons survived microinjection.

2.2.3.2 Analysis by immunofluorescence

Immunofluorescence was performed 20-24 hours after microinjection. All steps were carried out at room temperature and all rinses done three times using PBS unless otherwise stated. Coverslips were washed to remove SCG growth medium. Neurons were fixed in 4% paraformaldehyde for 30 minutes before being rinsed and then permeabilized with 0.5% Triton X-100 in PBS for 5 minutes. After rinsing, the cells were blocked using 50% horse serum in PBS for 30 minutes. The blocking solution was washed from the coverslips before incubation for one hour with the primary antibody, either a goat anti-luciferase polyclonal antibody or a goat anti-E4BP4 polyclonal antibody, both diluted 1:100 in PBS containing 10% horse serum. The neurons were rinsed and incubated in the dark for one hour with FITC-conjugated anti-goat IgG and rhodamine-conjugated anti-guinea pig IgG antibodies, both diluted 1:100 in PBS containing 10% horse serum. Following this secondary staining, the neurons were washed and the nuclei stained for 5 minutes with Hoechst dye at 10 μg/ml in water. A final rinse was done in water before the coverslips were air dried and mounted on glass slides in Citifluor, using clear nail varnish to seal the edges.

Slides were examined on a fluorescence microscope (model Axioplan 2; Carl Zeiss Ltd) and images captured with a digital camera (Photometrix Quantix) using SmartCapture VP software. The total number of injected neurons was scored, as well as the number of injected cells expressing luciferase at a level higher than the background fluorescence seen for uninjected cells. Experiments were scored in a blinded manner and each was performed at least three times.

2.2.3.3 Analysis by luciferase assay

Luciferase assays with microinjected sympathetic neurons were carried out at 16-24 hours after injection using the Dual-Luciferase® Reporter Assay system. Neurons were washed off the coverslips using chilled PBS and collected in a 1.5 ml microcentrifuge tube. The cells were centrifuged at 16,000 x g for 5 minutes at room temperature and the supernatant removed. The cell pellet was lysed in 25 µl of Passive Lysis buffer for 20 minutes at room temperature and vortexed on addition of the lysis buffer, after 10 minutes and at the end of the 20 minutes. The luciferase assay was then performed on 20 µl of the lysed neurons using a Lumat LB 9507 luminometer following the protocol provided with the assay system. To summarise, 95 µl of Luciferase Assay Reagent II was initially added to generate a firefly luciferase output that could be measured. This was followed by the addition of 95 µl of Stop & Glo® reagent to simultaneously quench firefly luciferase activity and initiate the *Renilla* luciferase reaction. The output for firefly luciferase was normalised to the *Renilla* luciferase output, controlling for variations in the amount of construct injected per neuron and the number of neurons injected. One-tailed paired or unpaired *t*-tests, depending on the dataset, were performed where appropriate using Microsoft Excel.

2.2.4 Transient transfection

2.2.4.1 Transient transfection of PC6.3 cells

Before transient transfection, differentiated PC6.3 cells were replated in 24 well plates at a density of $4x10^4$ cells per well 1-2 days before transfection. Neuronal PC6.3 cells were

transiently transfected 5-7 days after differentiation and were refed with antibiotic-free medium immediately prior to transfection. DNA reporter constructs and expression vectors were transfected using Lipofectamine™ 2000 according to individual experiments and at the concentrations indicated (see results chapters), typically 0.1-1 μg of reporter together with 50 ng of pRL-TK per well, mixed with 50 μl Opti-MEM. Lipofectamine™ 2000 (1 μl per well) was pre-mixed with Opti-MEM (50 μl per well) and incubated for 5-30 minutes at room temperature, before being gently mixed with the DNA to be transfected. After a further incubation at room temperature for 30 minutes, the transfection mixture was added to the cell medium in the wells. Cells were carefully refed with fresh complete medium 4-6 hours after transfection. Neuronally differentiated PC6.3 cells were transfected with ~5% efficiency.

2.2.4.2 Analysis by luciferase assay

Luciferase assays on PC6.3 cells were carried out at 24 hours after transfection using the Dual-Luciferase® Reporter Assay system. The medium was aspirated off the cells and 100 μl of Passive Lysis buffer was added to each well. After 20 minutes of gentle shaking at room temperature the luciferase assay was performed using 20 μl of cell lysate for each assay and following the protocol provided (see Section 2.2.3.3). Luminescence was measured using a Lumat LB 9507 luminometer. The firefly output was normalised to the *Renilla* output to control for variations in transfection efficiency.

2.2.5 RNA manipulations

2.2.5.1 RNA extraction

To harvest sympathetic neurons, the cells were rinsed from coverslips using ice cold PBS and were spun in 1.5 ml tubes at 16,100 x g for 5 minutes at 4°C. The supernatant was removed from the pelleted cells and RNA was extracted using an RNeasy® Mini kit following the protocol provided. In this procedure cells were lysed and homogenised by addition of 350 μl of a highly denaturing guanidine isothiocyanate (GITC)-containing buffer, buffer RLT, which inactivated RNases. The addition of ethanol provides the

appropriate binding conditions and the sample was applied and bound to the RNeasy silica-gel membrane. Contaminants were washed from the bound RNA which was finally eluted in 30 µl of RNase-free water and 40 units of recombinant RNasin® ribonuclease inhibitor were added.

2.2.5.2 Reverse transcriptase-PCR (RT-PCR)

RNA samples to be used in RT-PCR with primers for E4BP4 were DNase-treated using amplification grade Deoxyribonuclease I. 1x DNase I reaction buffer and 3.6 units of DNase I were added to the 30 µl of eluted RNA and then incubated at room temperature for 15 minutes. The DNase I was inactivated by the addition of 1 µl of 25 mM EDTA and by heating at 65°C for 10 minutes.

First strand cDNA was reverse transcribed using Superscript™ II Reverse Transcriptase following the manufacturer's instructions and using 10 µl of total RNA. 20 units of recombinant RNasin® ribonuclease inhibitor were added to each sample, rather than RNaseOUT™. A control for each sample was also made, in which no Superscript™ II RT was added (-RT samples). All reactions were inactivated by heating at 70°C for 15 minutes.

PCR reactions (50 µl) were prepared using 1-4 µl of cDNA with 1x REDTaq™ PCR Reaction Buffer, 0.1 µg of each primer (see section 2.1.4), 0.2 mM of each dNTP and 2.5 units of REDTaq™ DNA Polymerase. For PCR an initial denaturation of 2 minutes at 94°C and a final extension of 2 minutes at 72°C were used. The cycling parameters were as follows; for *dp5* and neurofilament (*nfm*) reactions cycles of 94°C for 30 seconds, 58°C for 20 seconds and 72°C for 1 minute; for *e4bp4*, *nfm* and *gapdh* reactions cycles of 94°C for 30 seconds, 59°C (*e4bp4* and *nfm*) or 54°C (*gapdh*) for 30 seconds and 72°C for 30 seconds. PCR cycles were performed a sufficient number of times for a product to be detected for each sample in a set, where possible. The amplified products were separated on a 2.5% agarose gel containing ethidium bromide to allow visualisation of the cDNA. Images were captured using an ImageMaster VDS-CL and quantified using ImageMaster Total Lab software.

2.2.6 Protein analysis

2.2.6.1 Polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was perfomed using the Bio-Rad Mini-PROTEAN 3 electrophoresis system. Protein samples and a Kaleidoscope prestained molecular weight marker were made up to 20 µl with TE and 1x SDS sample buffer and were heated at 90°C for 5 minutes. The samples were loaded on to the stacking gel and run on a 10% resolving gel at 200 V in 1x running buffer until the bromophenol blue dye had reached the bottom of the gel. The gel was immunoblotted as described below.

2.2.6.2 Immunoblotting

Following electrophoresis, proteins were transferred from the polyacrylamide gel to Hybond ECL nitrocellulose membrane using a BioRad Trans-Blot® transfer cell. The cassette containing the gel and membrane was carefully constructed, submerged in 1x transfer buffer and ran at 400 mA for 1.75 hours. After this time, successful transfer of the proteins was confirmed by staining the membrane for 5 minutes with 0.2% Ponceau-S solution and destaining in water. The stain was removed by rinsing in 1x TBST and the membrane was blocked overnight in 5% milk/TBS-T. The membrane was incubated for 1 hour with a 1:100-1:400 dilution of E4BP4 (V-19) goat polyclonal antibody in 5% milk/TBS-T with rotation. After one quick rinse, the membrane was washed three times with 5% milk/TBS-T for 10 minutes, and then incubated for 1 hour with a 1:1000 dilution of HRP-conjugated donkey-anti-goat antibody in 5% milk/TBS-T with rotation. The membrane was rinsed with 5% milk/TBS-T and then had three longer 10 minute washes before a final rinse in TBS-T. The presence of protein-bound antibody on the membrane was detected using ECL or ECL-plus, following the manufacturer's instructions and visualised by exposing the blot to Hyperfilm™ x-ray film.

2.2.7 DNA binding assay

2.2.7.1 *In vitro* translation of protein

E4BP4 protein, and a pcDNA3.1 control, was *in vitro* translated using a TNT® T7 coupled reticulocyte lysate system from a construct generated by Dr. Jenny Yeung, following the protocol provided. 50 µl reactions were prepared using 25 µl of TNT® rabbit reticulocyte lysate, 2 µl of TNT® reaction buffer, 1 µl of TNT® T7 RNA polymerase, 1 µl of 1 mM complete amino acid mix, 40 units of RNasin® ribonuclease inhibitor and 1 µg of template DNA. The reactions were left at 30°C for 60 minutes and 50 µl of 2x dialysis buffer was added and the reaction aliquoted for freezing. Successful translation of the protein was confirmed by immunoblotting.

2.2.7.2 Electrophoretic mobility shift assay (EMSA)

Single stranded oligonucleotides corresponding to the *dp5* sequence containing a potential E4BP4-binding site, as well as oligonucleotides containing three point mutations within this site, were annealed by mixing 2 µg of each with 10 µl of TM buffer in a total volume of 100 µl, to give a final concentration of 40 ng of double stranded probe per µl. These were heated at 80°C for 10 minutes and slow cooled to room temperature. The probes were radiolabelled by mixing 80 ng of each with 1 µl of Klenow polymerase, 1 µl of TM buffer, 1 mM of dGTP, dATP and dTTP and 2 µl [α-^{32}P]dCTP (3000 Ci/mMole; 10 µl total volume) and were incubated at room temperature for 15 minutes before being diluted to 0.4 ng of probe/µl in TE. The binding assay conditions were based on those described by Cowell *et al* (1992). Binding reactions (19 µl) were prepared with 1x E4 binding buffer, 10 of µg BSA, 1 µg of poly(I-C) and either 1, 2 or 4 µl of *in vitro* translated protein and were incubated at room temperature for 15 minutes. 0.4 ng (1 µl) of ^{32}P labelled probe was added before a further 15 minute incubation at room temperature. The binding reactions, as well as a sample containing 1x gel loading dye to allow tracking, were loaded on to a 6% acrylamide/0.5x TBE gel, which had been pre-run at 160 V for 30 minutes. The gel was run at 200 V until the samples had migrated ~8.5 cm and then the gel was fixed in 10% acetic acid/10%

methanol for 15 minutes. The fixed gel was placed on Whatman 3MM paper, covered with Saranwrap and dried using a BioRad gel dryer (model 583) for 2 hours. The bands were visualised by exposing the dried gel to Hyperfilm™ x-ray film or a phosphorimager screen overnight. The exposed phosphorimager screen was scanned using a Typhoon™ 8600 phosphorimager and the image analysed using ImageQuant software.

3 Characterisation of the rat *dp5* gene

3.1 Introduction

Sympathetic neurons are a well established model of developmental neuronal apoptosis. These cells depend on nerve growth factor (NGF) for survival during late embryonic and early postnatal development, and when cultured *in vitro*, undergo apoptosis if NGF is removed from the growth medium (Deshmukh and Johnson, Jr., 1997). This cell death displays many of the classic morphological features of apoptosis including shrinkage of the cell body and plasma membrane blebbing, as illustrated in Figure 3.1. Neuronal apoptosis is a complex and tightly controlled process involving the interaction of many regulatory proteins and can be blocked by inhibitors of transcription and translation (Martin *et al.*, 1988) suggesting that both *de novo* transcription and protein synthesis are required for this process to occur. The proapoptotic BH3-only protein DP5 is a BCL-2 family member that regulates neuronal apoptosis and DP5 expression is controlled at the transcriptional level. DP5 was first implicated in neuronal cell death when *dp5* mRNA levels were shown to increase in cultured sympathetic neurons undergoing apoptosis after NGF deprivation (Imaizumi *et al.*, 1997). The mRNA levels peak at 15 hours after NGF withdrawal, concurrent with the transcriptional commitment point for apoptosis in sympathetic neurons. These findings suggest that the transcriptional control of DP5 is important for its regulation but unlike other members of the BH3-only subfamily, such as BIM, little is known about the regulatory mechanisms involved.

Gene expression can be controlled through the regulation of mRNA level by either controlling the rate of RNA production or degradation. mRNA production is normally dependent on the DNA promoter sequence immediately upstream of the transcriptional start site but higher eukaryotic genes may also contain enhancers or silencers, regions of DNA sequence that control promoter activity through the binding of transcription factors and other gene regulatory proteins. These sequences can be located both 5' and 3' to the gene that they regulate, as well as within introns, and may be thousands of nucleotides away from the promoter (Levine and Tjian, 2003). For example, the 3' untranslated region (UTR) of the human *dp5* homologue *hrk* contains a

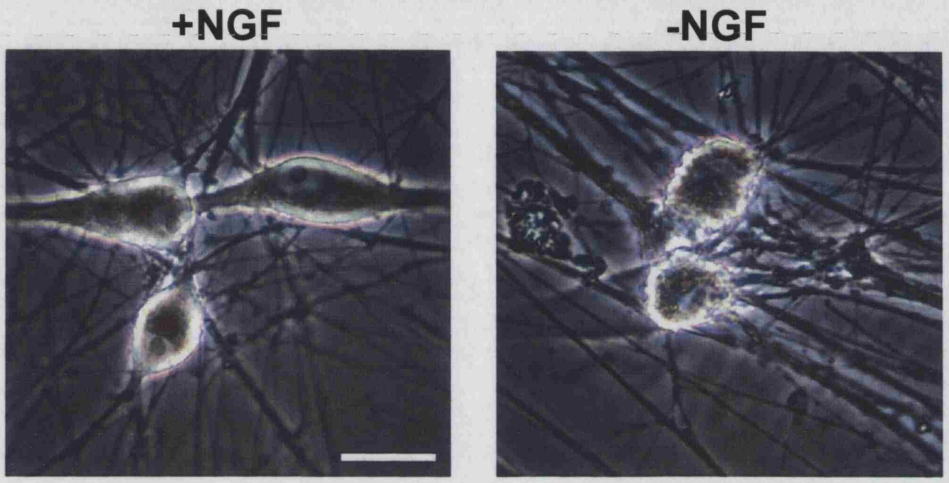


Figure 3.1 Morphology of sympathetic neurons cultured in the presence or absence of NGF

Sympathetic neurons were isolated from 1-day old rats and cultured in NGF-containing medium for 7 days. The cells were then cultured in the presence or absence of NGF for a further 48 hours before their morphology was examined using an Axiovert 200 phase-contrast microscope. Cells maintained in the presence of NGF have a normal spread morphology with a large nucleus and defined nucleoli, unlike those deprived of NGF, which have a shrunken, rounded morphology with a highly blebbing membrane, typical of apoptotic cells. The bar represents 20 μm.

silencer sequence that binds the transcriptional repressor DREAM, which prevents inappropriate *hrk* transcription and apoptosis in murine interleukin-3 (IL-3)-dependent haematopoietic cells (Sanz *et al.*, 2001). This type of regulatory element is able to modulate promoter activity if present on either DNA strand and in either orientation.

Gene expression can also be controlled by an alteration in the level of mRNA stability and consequently degradation. The RNA itself may contain a specific sequence that triggers degradation, such as the presence of an adenylate and uridylate-rich (AU-rich) element (ARE), often in multiple copies, found in the 3'UTR of many unstable RNAs. The 3'UTR of the antiapoptotic gene *bcl*-2 contains a destabilising ARE, a feature that is conserved from the *C. elegans* homologue *ced-9* through to the human *bcl-2* gene. The destabilising effect of the *bcl-2* ARE is enhanced in Jurkat cells by apoptotic stimuli, suggesting a role for this element in *bcl-2* down-regulation during apoptosis (Schiavone *et al.*, 2000). It is thought that such AU-rich sequences accelerate RNA degradation by triggering removal of the poly-A tail at the 3' end of the mRNA. It is also possible that the 3'UTR of some unstable mRNAs may contain recognition sites for endonucleases that cleave the RNA.

Both of these mechanisms for regulating mRNA level provide a means of conferring cell-type specificity of gene expression as well as expression in response to extracelluar stimuli. To understand the mechanism by which *dp5* gene expression is regulated by NGF in sympathetic neurons it is essential to identify the regions of the gene that contribute to this process and which result in increased *dp5* mRNA levels after NGF withdrawal. The rat *dp5* gene consists of two exons divided by a large 18.9 kb intron (Figure 3.2). Exon 1 contains the open reading frame (ORF) as well as a small region of 3'UTR (108 bp), the remainder of which is located in exon 2 (4882 bp). Despite the large size of the intron only a short region towards the 5' end is conserved between the rat, mouse and human sequences. In our laboratory, 4.5 kb of the promoter sequence upstream of the *dp5* transcriptional start site was cloned by Dr. Jonathan Gilley. To test whether these regions contained elements that responded to NGF, sequences from the promoter region, intron and 3'UTR were subcloned into luciferase reporter constructs, which were microinjected into sympathetic neurons to determine whether the addition of these sequences altered luciferase expression after NGF

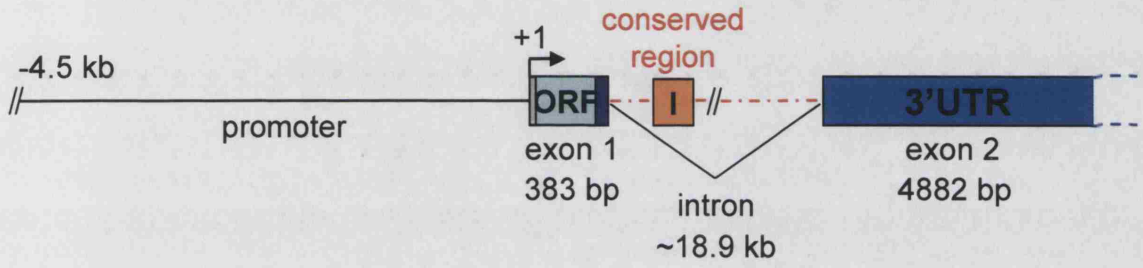


Figure 3.2 Structure of the rat *dp5* gene

The *dp5* gene consists of 2 exons and an 18.9 kb intron. The transcriptional start site mapped by 5' RACE is indicated as +1. Exon 1 is 383 bp long and contains the DP5 ORF as well as a small region of 3'UTR. Exon 2 is 4882 bp long and contains the 3'UTR. A small region (<400 bp) towards the 5' end of the intron is conserved in sequence between the rat, mouse and human *dp5* genes.

Figure 3.3 Microinjection of sympathetic neurons

A) Outline of the strategy for the culture and microinjection of sympathetic neurons.

B) In microinjection experiments, the luciferase reporter constructs being tested were microinjected directly into the nuclei of sympathetic neurons using needles pulled from glass capillary tubes loaded with the injection mix. Typically 150-200 neurons would be injected per test construct using an Eppendorf 5171 micromanipulator and 5272 transjector set to manual mode. It normally takes ~30 minutes to microinject 150 neurons. The bar represents 20 μm. The average diameter of a sympathetic neuron nucleus is ~10 μm.

A

sympathetic neurons obtained from SCG of P1 rats

↓

cultured *in vitro* for 5-7 days (+NGF)

↓

microinjected with test construct and either guinea pig IgG or *Renilla* luciferase construct

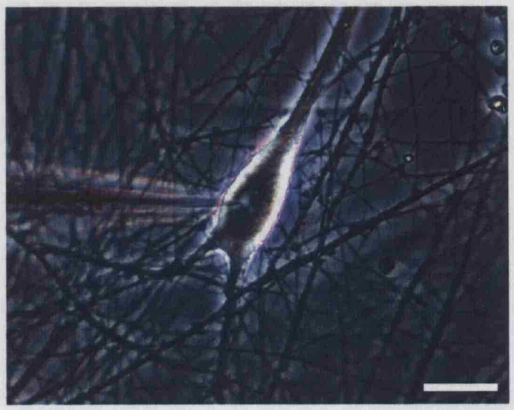
↓

16-24 hours in test conditions, e.g.+/-NGF

↓

luciferase activity determined by immunofluorescence or dual luciferase assay

B



withdrawal (Figure 3.3A). This strategy was successfully used in experiments with sympathetic neurons to localize important regulatory regions of the proapoptotic BH3-only gene *bim* (Gilley *et al.*, 2003;Gilley and Ham, 2005). By cloning regions of the gene of interest into reporter constructs containing firefly luciferase, alterations in the level of gene expression can be easily measured by either immunofluorescence or by a dual luciferase assay, in which firefly luciferase activity is compared to the activity of a control *Renilla* luciferase construct. With these assays, reproducible luciferase measurements can be made and are also sensitive enough to be effective in measuring luciferase activity in a relatively small number of cells, a restriction due to the limited number of cells that can be microinjected. Despite microinjection being a difficult and time consuming process it is a useful technique because it allows the direct delivery of DNA into the nucleus of primary neurons (Figure 3.3B), overcoming the problem of being unable to efficiently transfect the small number of cells that are obtained from P1 rat SCG (Garcia *et al.*, 1992;Pajak *et al.*, 2003).

The results of testing different *dp5* luciferase reporter constructs containing major regions of the *dp5* gene by microinjection are outlined in this chapter. Some repeat experiments were done in collaboration with my supervisor, Dr. Jonathan Ham.

3.2 Results

3.2.1 Measurement of the level of luciferase expression using a dual luciferase assay is comparable to scoring cells stained by immunofluorescence

Luciferase reporter constructs containing different regions of the *dp5* gene were microinjected into sympathetic neurons. To determine whether these regions contained elements that responded to NGF withdrawal the level of luciferase activity was measured and the level of luciferase expression in the presence and absence of NGF was compared. Preliminary work had been carried out using immunofluorescence to measure luciferase activity by injecting cells with the reporter construct to be tested as well as an injection marker (guinea pig IgG). After being stained, the number of microinjected neurons expressing luciferase was scored in a blinded manner and the

percentage of luciferase-expressing cells in the absence of NGF (-NGF) was calculated relative to the percentage expressing luciferase in the presence of NGF (+NGF). More than 50% of the injected neurons survived this process. As illustrated in Figure 3.4A an increase in luciferase expression could be visualized if a construct was activated by NGF deprivation but there were some limitations in using this semi-quantitative technique. Although the percentage of injected cells with levels of luciferase expression above background could be scored, the level of expression, which varied between injected cells (Figure 3.4Aiii), could not be measured.

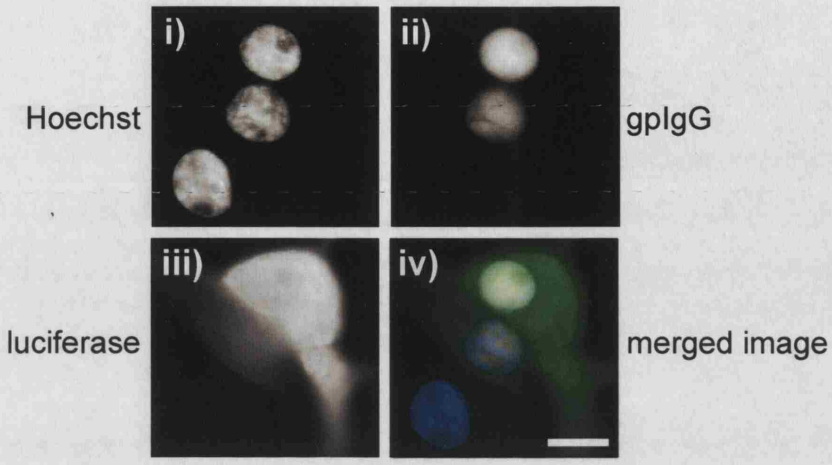
To overcome this problem, the dual luciferase assay technique was adapted for microinjected neurons. This method required cells to be co-injected with a *Renilla* luciferase-expressing construct as well as the firefly luciferase reporter construct to be tested. Firefly luciferase activity was then measured using a luminometer and was calculated relative to *Renilla* luciferase activity to control for variations in the amount of construct injected per neuron and the number of neurons injected. Once again, luciferase activity -NGF was calculated relative to activity +NGF. Figure 3.4B shows that both of these methods for measuring luciferase expression gave comparable results when they were used to test the same pair of constructs. In both cases construct *Y* (1kb*dp5*-LUC+3'UTR, see Section 3.2.3) showed a greater increase in luciferase activity after NGF withdrawal compared to construct *X* (1kb*dp5*-LUC, see Section 3.2.2). This figure also illustrates the benefit of using the dual luciferase assay because with this method there is an increased sensitivity for measuring luciferase activity shown by the higher level of induction after NGF deprivation for both constructs compared to analysis by immunofluorescence. In this fully quantitative and less time-consuming assay an increase in luciferase activity could be measured where previously only a negligible induction was detected for construct *X* (Figure 3.4B). In addition, fewer cells needed to be injected and background measurements for uninjected cells were comparable to measuring sample-free reaction reagents, indicating that this technique was suitable for determining luciferase activity in this system and it was used in all subsequent experiments.

Figure 3.4 Comparison of immunofluorescence and the dual luciferase assay as methods for measuring luciferase expression in microinjected sympathetic neurons

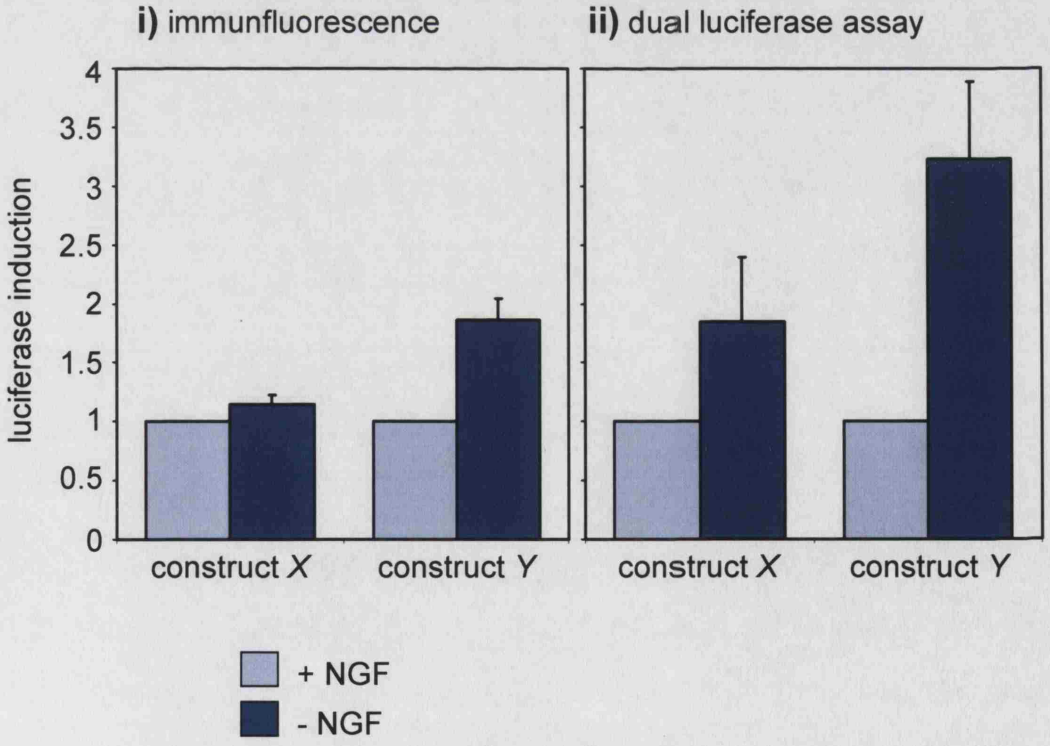
A) An example of sympathetic neurons microinjected with a reporter construct that is activated by NGF withdrawal and which were analysed by immunofluorescence. In this example sympathetic neurons were microinjected with 1kbdp5-LUC+A(F) [0.009 μg/μl] together with gpIgG [2.5 μg/μl] and were maintained in the absence of NGF for 24 hours before lucferase expression was measured. **i)** Hoechst staining of all nuclei, **ii)** guinea pig IgG staining identifies microinjected cells, **iii)** injected cells demonstrate varying levels of luciferase expression, **iv)** merged image of all three colours. The bar represents 10 μm.

B) An example of a series of experiments in which sympathetic neurons were microinjected with the same set of constructs and luciferase expression was analysed by either **i)** immunofluorescence or **ii)** dual luciferase assay. Construct *X* (1kb*dp5*-LUC [0.005 μg/μl]) or construct *Y* (1kb*dp5*-LUC+3'UTR [0.015 μg/μl]) were injected together with **i)** gpIgG [2.5 μg/μl] or **ii)** a *Renilla* luciferase expression vector [0.005 μg/μl]. The neurons were then maintained for 16-24 hours +/- NGF. Luciferase induction was determined as follows. The level of luciferase expression for each construct was measured by **i)** scoring the percentage of injected neurons (as determined by the presence of gpIgG) expressing the luciferase protein or **ii)** measuring the level of firefly luciferase activity on a luminometer and normalising this for *Renilla* luciferase activity (firefly activity ÷ *Renilla* luciferase activity). The relative luciferase induction was calculated by setting the luciferase expression/activity in the presence of NGF as 1 and then calculating the induction in the absence NGF relative to this (expression or activity –NGF ÷ expression or activity +NGF). The mean values for 4 experiments ± the standard error of the mean (SEM) are shown.

A


i)
ii)
Hoechst
gpIgG
iii)
iv)
luciferase
merged image

B


i) immunfluorescence
ii) dual luciferase assay
luciferase induction
4
3.5
3
2.5
2
1.5
1
0.5
0
construct X
construct Y
construct X
construct Y
+ NGF
- NGF

3.2.2 1kb of the *dp5* promoter region contains an element that responds to NGF

The first region of the *dp5* gene tested was the 1 kb sequence immediately upstream of the transcriptional start site. This DNA sequence was inserted directly upstream of luciferase in a promoterless reporter construct (1kb*dp5*-LUC, Figure 3.5A). The response of this region of *dp5* to NGF deprivation was tested in a series of microinjection experiments in which sympathetic neurons were cultured for 5 to 7 days *in vitro* and then 150 cells per coverslip were microinjected with this construct, as well as the control *Renilla* luciferase construct (pRL-TK). Microinjected neurons were maintained in the presence or absence of NGF for 16 to 24 hours, after which time the level of luciferase activity was measured using the dual luciferase assay and the relative luciferase induction was calculated as described in Section 3.2.1. Throughout the series of experiments described in this chapter this construct containing 1 kb of the promoter region was used as a comparison for most other *dp5* reporter constructs. The spread of induction for 1kb*dp5*-LUC after NGF withdrawal (luciferase activity –NGF ÷ luciferase activity +NGF) for 40 experiments is shown in Figure 3.5B. Despite there being some variation in the fold induction of this construct after NGF withdrawal, the inter-quartile range was limited to between 1.36 and 2.61 with a mean induction for this construct of 2.03. An induction of this level suggests that this 1 kb of promoter region contains elements that increase *dp5* promoter activity after NGF withdrawal.

To localise the region of the 1kb promoter sequence that contained the NGF-responsive element 1kb*dp5*-LUC was compared to a similar construct that contained only 150 bp of the sequence immediately upstream of the *dp5* transcriptional start site (150bp*dp5*-LUC, Figure 3.5C). When tested as above, the construct containing the shorter 150 bp region of promoter DNA had a significantly reduced response after NGF withdrawal compared to the full 1 kb. This result suggests that this 150 bp alone does not contain a region that responds to NGF, or that it is not sufficient on its own to cause the induction obtained when 1 kb is present.

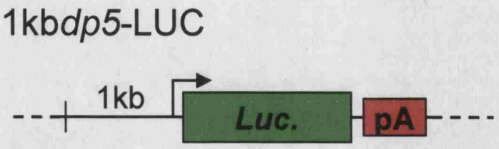
Figure 3.5 The 1 kb *dp5* promoter contains sequences that respond to NGF withdrawal

A) A *dp5* luciferase construct was made containing 1kb of the *dp5* promoter sequence upstream of luciferase. The reporter construct also contained the SV40 poly-A (pA) termination signal.

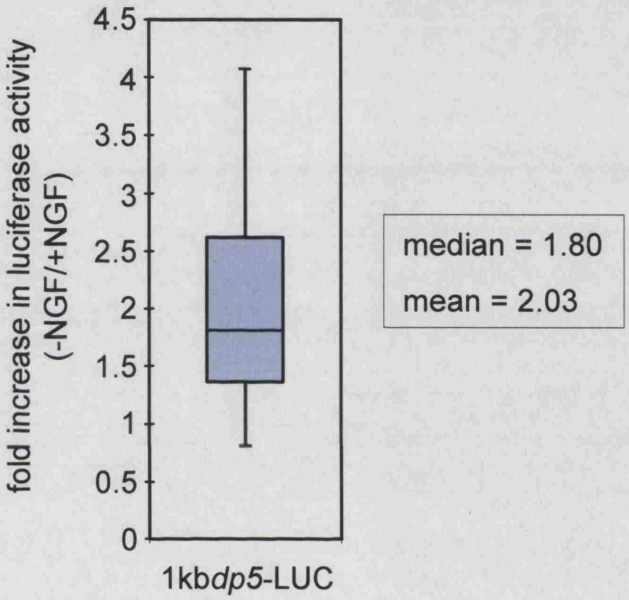
B) Sympathetic neurons were microinjected with 1kb*dp5*-LUC [0.005 μg/μl]. The neurons were maintained for 16-24 hours +/- NGF and luciferase activity was determined using the dual luciferase assay. Luciferase activity –NGF was calculated relative to activity +NGF. The results from 40 experiments are plotted. The box represents the interquartile range and the whiskers represent the upper and lower quartiles. The median value is marked on the plot.

C) Sympathetic neurons were microinjected with 1kb*dp5*-LUC [0.005 μg/μl] or 150bp*dp5*-LUC [0.0043 μg/μl]. The neurons were maintained for 20-24 hours +/- NGF and luciferase activity was determined using the dual luciferase assay. Luciferase activity –NGF was calculated relative to activity +NGF. The mean of 6 experiments ± the SEM is shown (* $P<0.05$, *t*-test).

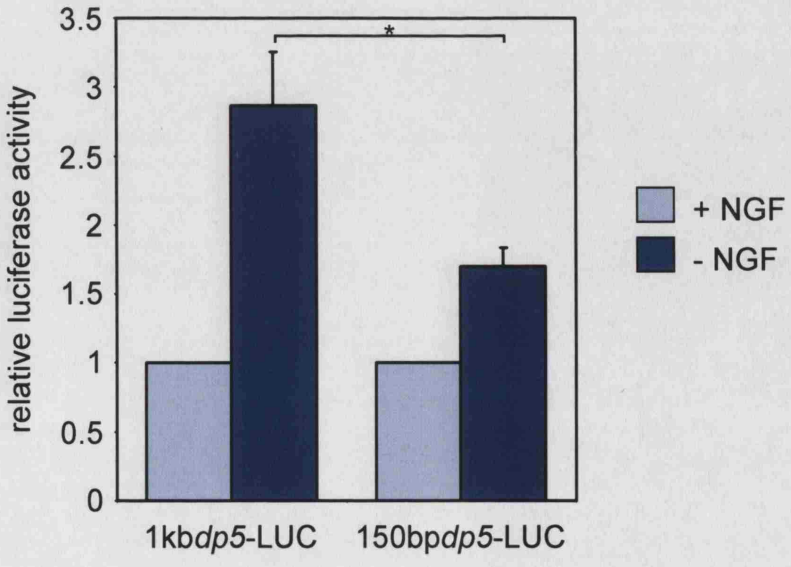
A


1kbdp5-LUC
1kb
Luc.
pA

B


fold increase in luciferase activity
(-NGF/+NGF)
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
1kbdp5-LUC
median = 1.80
mean = 2.03

C


relative luciferase activity
3.5
3
2.5
2
1.5
1
0.5
0
*
1kbdp5-LUC
150bpdp5-LUC
+ NGF
- NGF

3.2.3 The *dp5* intron and 3'UTR contain elements that respond to NGF deprivation in sympathetic neurons

Because transcript levels can be regulated by sequences upstream or downstream of a promoter region, it was important to determine whether addition of other regions of the *dp5* gene altered *dp5* promoter activity or *dp5* mRNA stability. To test this, luciferase reporter constructs were made that contained DNA sequences from the *dp5* intron and/or the *dp5* 3'UTR and were microinjected into sympathetic neurons. The *dp5* intron that separates exon 1 and 2 is ~18.9 kb long but only ~400 bp of this is conserved between the rat, mouse and human sequences. Functionally important DNA often shows high levels of sequence conservation across species suggesting that this region could be a potential candidate for containing a regulatory element that responded to NGF deprivation. To test this, a ~400 bp DNA fragment containing most of this conserved region of the intron (fragment I) was subcloned into a luciferase reporter construct that also contained 1 kb of the *dp5* promoter (1kb*dp5*-LUC+I(F), Figure 3.6Ai). Fragment I was inserted downstream of the poly-A termination signal because this would also determine whether any effect seen by addition of this sequence was due to the presence of an enhancer, which should function independently of position and orientation. This construct was microinjected into sympathetic neurons and the level of luciferase activity after NGF withdrawal was measured as described above. In a similar manner to the reporter construct containing the *dp5* promoter region, 1kb*dp5*-LUC+I(F) showed an increase in luciferase activity after NGF withdrawal, resulting in a 3.61 fold induction after NGF deprivation (Figure 3.6Bi). The addition of fragment I caused a significant increase in luciferase induction compared to 1kb*dp5*-LUC ($p<0.01$), suggesting that this relatively short DNA sequence from the conserved region of the *dp5* intron contains an enhancer that responds to NGF withdrawal in sympathetic neurons to increase the promoter activity.

The *dp5* 3'UTR is 5.025 kb long and although it is spilt between two exons only 108 bp is located in exon 1 with the remainder comprising exon 2. To test whether this region contained any elements that alter *dp5* promoter activity or mRNA stability, the entire 3'UTR was subcloned downstream of luciferase in a construct that also contained 1 kb of the promoter sequence (1kb*dp5*-LUC+3'UTR, Figure 3.6Aii). This construct

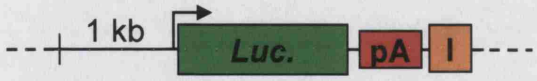
Figure 3.6 The *dp5* intron and 3'UTR contain sequences that respond to NGF withdrawal

A) *dp5* luciferase constructs were made that contained 1 kb of the *dp5* promoter sequence upstream of the firefly luciferase gene in pGL3-basic and either **i)** ~400 bp of the *dp5* intron sequence that is conserved between rat, mouse and human sequences, inserted downstream of the poly(A) termination signal (1kb*dp5*-LUC+I(F)), **ii)** the *dp5* 3'UTR inserted downstream of luciferase (1kb*dp5*-LUC+3'UTR) or **iii)** both the intron fragment and 3'UTR inserted as above (1kb*dp5*-LUC+ALL). All luciferase reporter constructs contain the SV40 poly-A transcription termination signal.

B) Sympathetic neurons were microinjected with 1kb*dp5*-LUC [0.005 μg/μl] or either **i)** 1kb*dp5*-LUC+I(F) [0.005 μg/μl], **ii)** 1kb*dp5*-LUC+3'UTR [0.015 μg/μl] or **iii)** 1kb*dp5*-LUC+ALL [0.01 μg/μl]. The neurons were maintained for 20-24 hours +/- NGF and luciferase activity was determined using the dual luciferase assay. Luciferase activity –NGF was calculated relative to activity +NGF. The mean of at least 4 experiments ± the SEM is shown (* $P<0.05$, ** $P<0.01$, *t*-test).

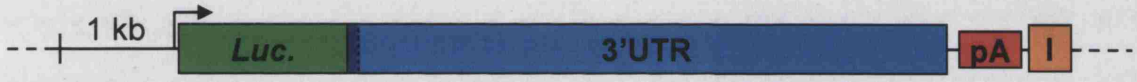
A

i) 1kb*dp5*-LUC+I(F)

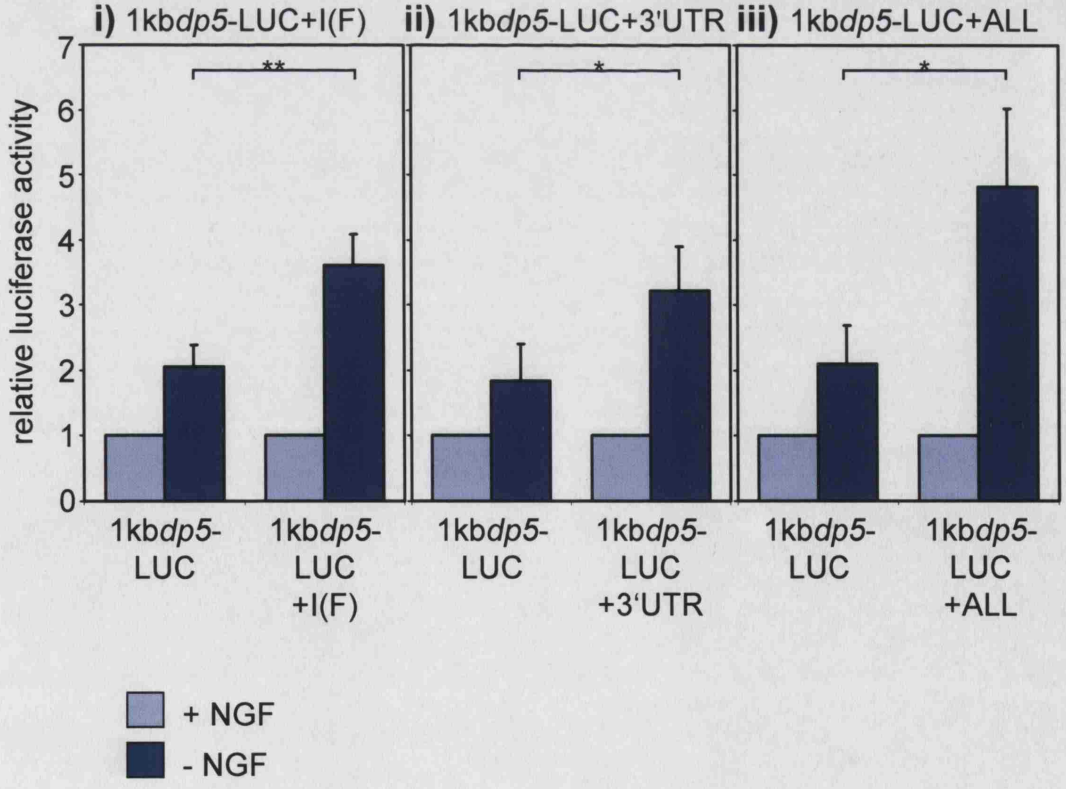


ii) 1kb*dp5*-LUC+3'UTR

iii) 1kb*dp5*-LUC+ALL



B



was microinjected into sympathetic neurons and its activity in the presence and absence of NGF was determined. The activity of 1kb*dp5*-LUC+3'UTR increased after NGF withdrawal and the addition of the 3'UTR to the *dp5* promoter resulted in a significantly larger induction of luciferase (3.23 fold) compared to the promoter alone (1.84 fold, $p<0.05$). This further increase in luciferase induction measured in the absence of NGF was comparable to that seen with 1kb*dp5*-LUC+I(F), since both of these constructs had an induction factor 75% greater than that of 1kb*dp5*-LUC. This result suggests that the 3'UTR contains a regulatory region that causes an increase in *dp5* expression after NGF deprivation in sympathetic neurons.

Constructs containing either the *dp5* intron fragment or the 3'UTR region were both activated after NGF withdrawal and in both cases the inclusion of either the intron fragment or the 3'UTR region, in addition to 1 kb of the promoter region, resulted in higher levels of induction compared to a construct containing the promoter alone. To investigate the effect of the presence of all three regions, a construct was made that contained the 1 kb promoter region, fragment I and the 3'UTR (1kb*dp5*-LUC+ALL, Figure 3.6Aiii) and this was tested by microinjection into sympathetic neurons. This construct was robustly activated after NGF withdrawal with a 4.81 fold induction relative to +NGF (Figure 3.6Biii). This level of induction was larger than that seen when either fragment I or the 3'UTR were present separately and was 129% greater than the induction seen with the 1kb*dp5*-LUC control ($p<0.05$). This further supports the hypothesis that each of these regions contributes to luciferase induction in response to NGF withdrawal because the highest level of induction is obtained when all three *dp5* regions are present together.

3.2.4 The *dp5* intron enhancer element functions in a context-specific manner

The increased induction measured when a luciferase reporter construct containing a region from the *dp5* intron as well as 1 kb of DNA sequence from the promoter suggests that this intron region contains an NGF responsive element. The intron fragment was inserted downstream of the poly-A termination signal, which implies that this fragment

functions as an enhancer because these elements function independently of location. To examine whether fragment I has a similar enhancing effect on a heterologous promoter, the intron sequence was inserted into a luciferase reporter construct upstream of the SV40 promoter (SV40-LUC+I(F), Figure 3.7A) and either this construct or a construct containing only the SV40 promoter (pGL3-P) was microinjected into sympathetic neurons. The results show that microinjecting the construct containing only the SV40 promoter did not result in a substantial increase in luciferase activity after NGF withdrawal (1.39 fold) as predicted because this promoter is not known to be activated by NGF deprivation. Addition of the *dp5* fragment I sequence to this construct did not alter SV40 promoter activity because the induction measured after NGF withdrawal was comparable to that of the SV40 promoter alone (1.48 fold, Figure 3.7B). This suggests that the *dp5* intron fragment did not increase the activity of this heterologous promoter in response to NGF deprivation and did not act as an enhancer in this context.

3.2.5 An NGF-responsive region was localized to 645 bp within the *dp5* 3'UTR

The addition of the entire *dp5* 3'UTR sequence to constructs containing the promoter and both the promoter and intron increased the response of these constructs to NGF deprivation, evident by the increased luciferase induction seen with constructs containing this 3'UTR. Despite the length of this sequence only a relatively small region is conserved between the rat, mouse and human *dp5/hrk* sequences, with the human 3'UTR being substantially shorter. Most of this sequence conservation is found towards the 5' end of the rat 3'UTR. The 3'UTR of the human homologue of *dp5*, *hrk*, contains a downstream regulatory element (DRE) in this region that has been shown to interact with a transcriptional repressor (DREAM), which is regulated by IL-3 in IL-3-dependent haematopoietic progenitor cells (Sanz *et al.*, 2001). Examination of the *dp5* sequence indicated that a similar DRE might be present in the 3'UTR of the rat *dp5* gene and so 645 bp from the 5' end of the 3'UTR, comprising the 3'UTR sequence from both exon 1 and exon 2 (fragment A), was inserted in the forward (5' to 3') orientation downstream of luciferase in a construct also containing 1 kb of the *dp5* promoter sequence (1kb*dp5*-

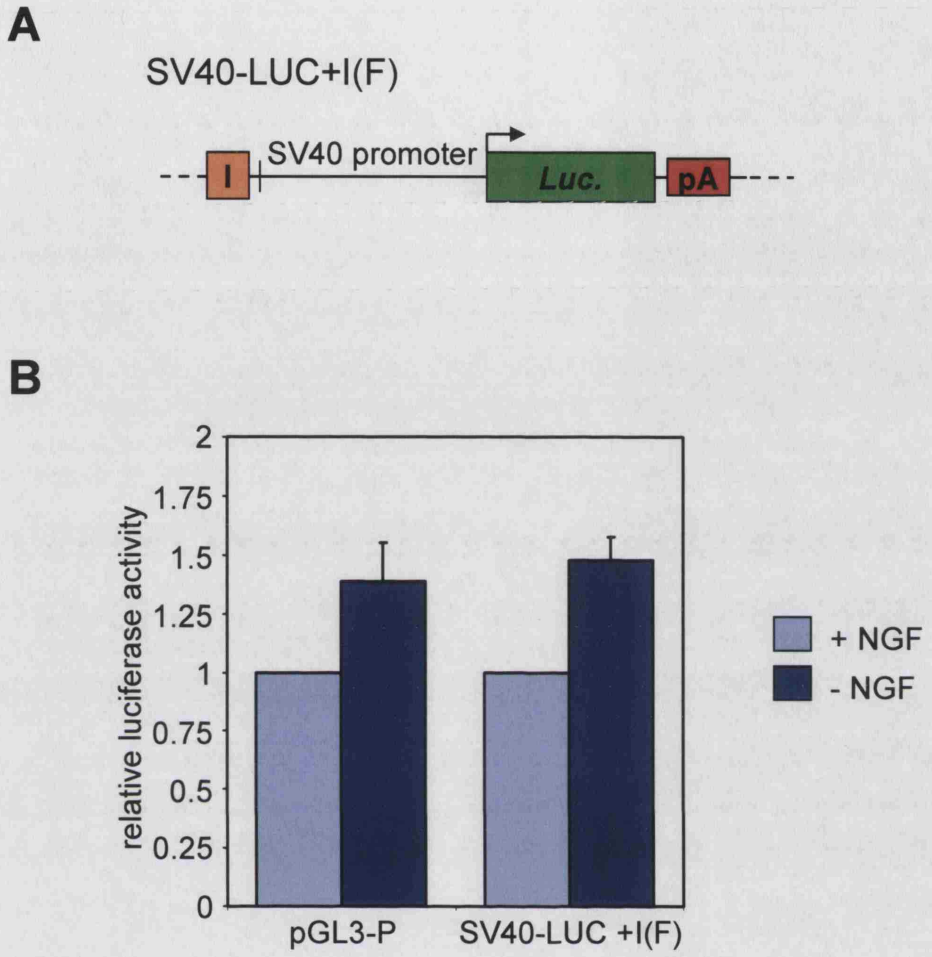


Figure 3.7 The conserved region of the *dp5* intron does not act as an enhancer for a heterologous promoter

A) A luciferase reporter construct was made containing ~400 bp of the *dp5* intron sequence that is conserved between rat, mouse and human sequences, inserted upstream of the SV40 promoter in pGL3-P (SV40-LUC+I(F)). This reporter construct also contains the SV40 poly-A transcription termination signal.

B) Sympathetic neurons were microinjected with pGL3-P [0.01 μg/μl] or SV40-LUC+I(F) [0.01 μg/μl]. The neurons were maintained for 20-24 hours +/- NGF and luciferase activity was determined using the dual luciferase assay. Luciferase activity –NGF was calculated relative to activity +NGF. The mean of 5 experiments ± the SEM is shown.

LUC+A(F), Figure 3.8A). This construct was tested by microinjection into sympathetic neurons and expression levels were compared to 1kb*dp5*-LUC. Initial experiments using both immunofluorescence and the dual luciferase assay to measure luciferase induction after NGF withdrawal indicated that this construct responded in a similar manner to 1kb*dp5*-LUC+3'UTR, which contains the whole of the *dp5* 3' untranslated region. In both of these experiments the presence of fragment A resulted in a higher level of induction compared to the promoter alone (Figure 3.8B) but there did appear to be a degree of variation between repeats of the experiment using the dual luciferase assay, illustrated by the large error bars representing the standard error of the mean. From these experiments it is difficult to interpret if the effect of adding fragment A is real or not, but because a significant effect was seen using immunofluorescence ($p<0.05$), this construct was tested more thoroughly.

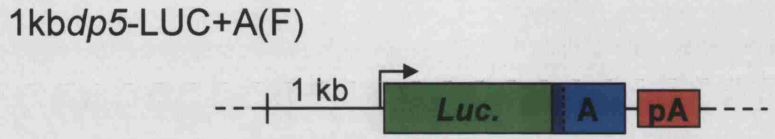
To try and identify a cause of the variation seen when testing 1kb*dp5*-LUC+A(F), the time after NGF withdrawal at which the dual luciferase assay was performed was varied. Previously, the assay was carried out at time points ranging between 16 and 24 hours after NGF was removed from the medium and it was possible that using different time points could account for the range of inductions measured. Therefore the responses of two different *dp5* luciferase reporter constructs, 1kb*dp5*-LUC and 1kb*dp5*-LUC+A(F), at 16, 20 and 24 hours after NGF deprivation were tested. In these experiments the time at which sympathetic neurons were microinjected and the time at which NGF withdrawal was carried out was staggered to ensure that the period between microinjection and NGF deprivation remained constant for each time point and to allow the luciferase assays to be performed together. Due to a restriction in the number of neurons that could be microinjected, the two constructs were tested in separate experiments. The luciferase induction in cells microinjected with 1kb*dp5*-LUC varied slightly between 16 and 24 hours, having the highest level of 2.4 at 20 hours (Figure 3.9A). With the construct 1kb*dp5*-LUC+A(F), the induction of luciferase expression on NGF deprivation increased over time, increasing ~1.7 fold between 16 and 24 hours, with an induction of 4.35 at this latest time point (Figure 3.9B). These results indicated that differences in the time cells are left in the presence and absence of NGF could cause variations in the luciferase expression measured. 20-24 hours was

Figure 3.8 A 645 bp fragment at the 5' end of the *dp5* 3'UTR (fragment A) contains sequences that respond to NGF withdrawal

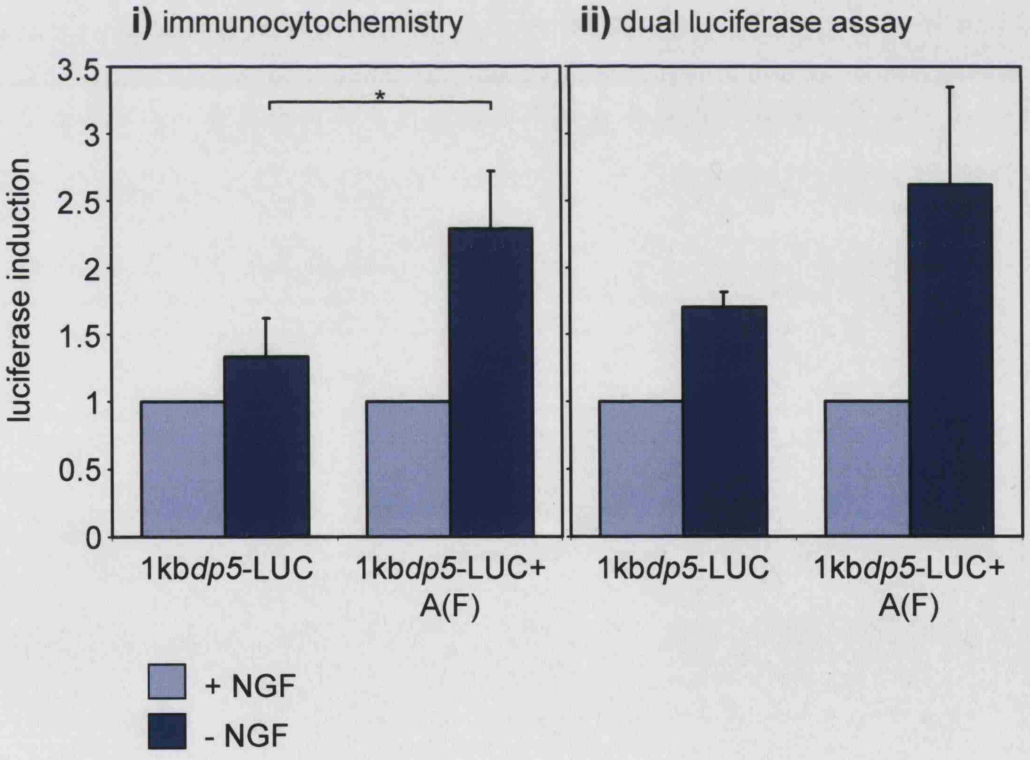
A) A *dp5* luciferase reporter construct was made containing 645 bp of *dp5* sequence from the 5' end of the 3'UTR downstream of the firefly luciferase gene and the 1 kb *dp5* promoter (1kb*dp5*-LUC+A(F)). The construct also contained the SV40 poly-A transcription termination signal.

B) Sympathetic neurons were microinjected with the 1kb*dp5*-LUC [0.005 μg/μl] or 1kb*dp5*-LUC+A(F) [0.009 μg/μl] construct, together with **i)** gpIgG [2.5 μg/μl] or **ii)** a *Renilla* luciferase expression vector [0.005 μg/μl]. The neurons were maintained for 16-24 hours in the presence or absence of NGF. Luciferase activity was determined by **i)** immunocytochemistry and **ii)** using the dual luciferase assay, and activity –NGF was calculated relative to activity +NGF. The mean of 4 experiments ± the SEM is shown (* $P<0.05$, *t*-test).

A


1kbdp5-LUC+A(F)
1 kb
Luc.
A
pA

B


i) immunocytochemistry
ii) dual luciferase assay
luciferase induction
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*
1kbdp5-LUC
1kbdp5-LUC+ A(F)
1kbdp5-LUC
1kbdp5-LUC+ A(F)
+ NGF
- NGF

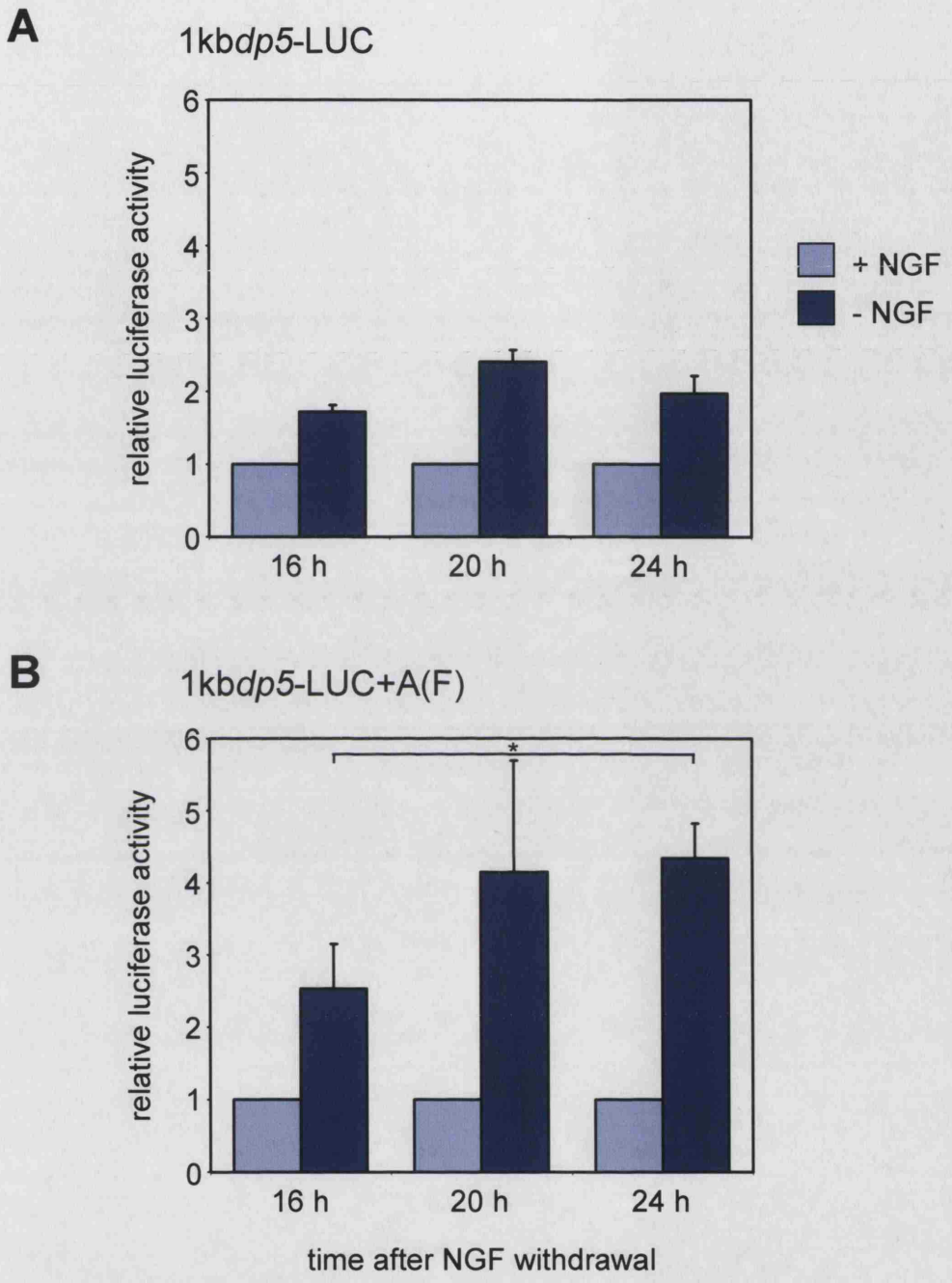


Figure 3.9 Comparison of *dp5* luciferase reporter constructs at different time points after NGF withdrawal

Sympathetic neurons were microinjected with **A)** 1kb*dp5*-luc [0.005 µg/µl] or **B)** 1kb*dp5*-luc+A(F) [0.009 µg/µl], together with a *Renilla* luciferase expression vector [0.005 µg/µl], and were maintained for 16, 20 and 24 hours +/- NGF. Luciferase activity was determined by using the dual luciferase assay, and activity –NGF was calculated relative to activity +NGF. The mean of 3 experiments ± the SEM is shown (* $P<0.05$, *t*-test).

selected as a suitable time after NGF withdrawal to assay for luciferase activity because this appeared to be the time at which levels of luciferase induction –NGF were the highest for these constructs. The observation that luciferase induction was higher at all of the time points –NGF for 1kb*dp5*-LUC+A(F) compared to 1kb*dp5*-LUC, combined with the previous results, suggests that fragment A does contain a *dp5* regulatory element that increases the response to NGF withdrawal. However the experiments shown in Figure 3.9A and B should not be directly compared because the constructs were tested in separate experiments.

3.2.6 DNA sequences from either side of the intron are required for fragment A to respond to NGF deprivation and may affect mRNA stability

The results obtained above suggest that a 645 bp region at the 5' end of the *dp5* 3'UTR is sufficient to cause an increase in the induction of luciferase after NGF deprivation. To localise exactly where in fragment A any regulatory regions are, a further two reporter constructs were made. Fragment A1 contains only the 109 bp of 3'UTR sequence from exon 1 and fragment A2 contains the 536 bp of fragment A from exon 2, including the potential DRE sequence. Both fragment A1 and A2 were inserted downstream of luciferase into reporter constructs also containing the 1kb of *dp5* sequence immediately upstream of the transcriptional start site (1kb*dp5*-LUC+A1 and 1kb*dp5*-LUC+A2, Figure 3.10Ai and ii). These constructs were microinjected into sympathetic neurons using 1kb*dp5*-LUC as a control, after which cells were maintained + and – NGF and luciferase activity was measured by dual luciferase assay. The addition of fragment A1 to the construct did not result in a large induction of luciferase after NGF withdrawal (1.32 fold, Figure 3.10Bi) and although the construct containing fragment A2 had a higher level of induction than A1 (1.92 fold, Figure 3.10Bii) in neither case was the level after NGF withdrawal higher than that obtained with 1kb*dp5*-LUC. This suggests that neither fragment A1 nor fragment A2 alone are able to enhance the response to NGF deprivation and it is unlikely that the potential DRE in fragment A2 acts as a regulatory element in sympathetic neurons.

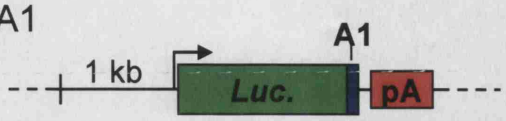
Figure 3.10 The response to NGF withdrawal requires *dp5* 3'UTR sequences from both sides of the intron and the response decreases when fragment A is in the reverse orientation

A) *dp5* luciferase reporter constructs were made containing 1kb of the *dp5* promoter sequence upstream of luciferase and either **i)** the 108 bp of 3'UTR from exon 1 (1kb*dp5*-LUC+A1) **ii)** the first 534 bp of 3'UTR from exon 2 (1kb*dp5*-LUC+A2) or **iii)** fragment A in the reverse (3'-5') orientation (1kb*dp5*-LUC+A(R)). All luciferase reporter constructs contain the SV40 poly-A transcription termination signal.

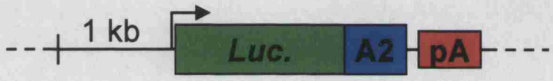
B) Sympathetic neurons were microinjected with 1kb*dp5*-LUC [0.005 μg/μl] or either **i)** 1kbbp*dp5*-LUC+A1 [0.005 μg/μl], **ii)** 1kb*dp5*-LUC+A2 [0.005 μg/μl] or **iii)** 1kb*dp5*-LUC+A(R) [0.009 μg/μl]. The neurons were maintained for 20-24 hours +/- NGF and luciferase activity was determined using the dual luciferase assay. Luciferase activity –NGF was calculated relative to activity +NGF. The mean of at least 3 experiments ± the SEM is shown.

A

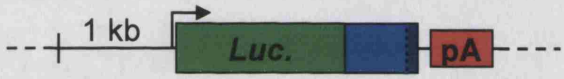
i) 1kb*dp5*-LUC+A1



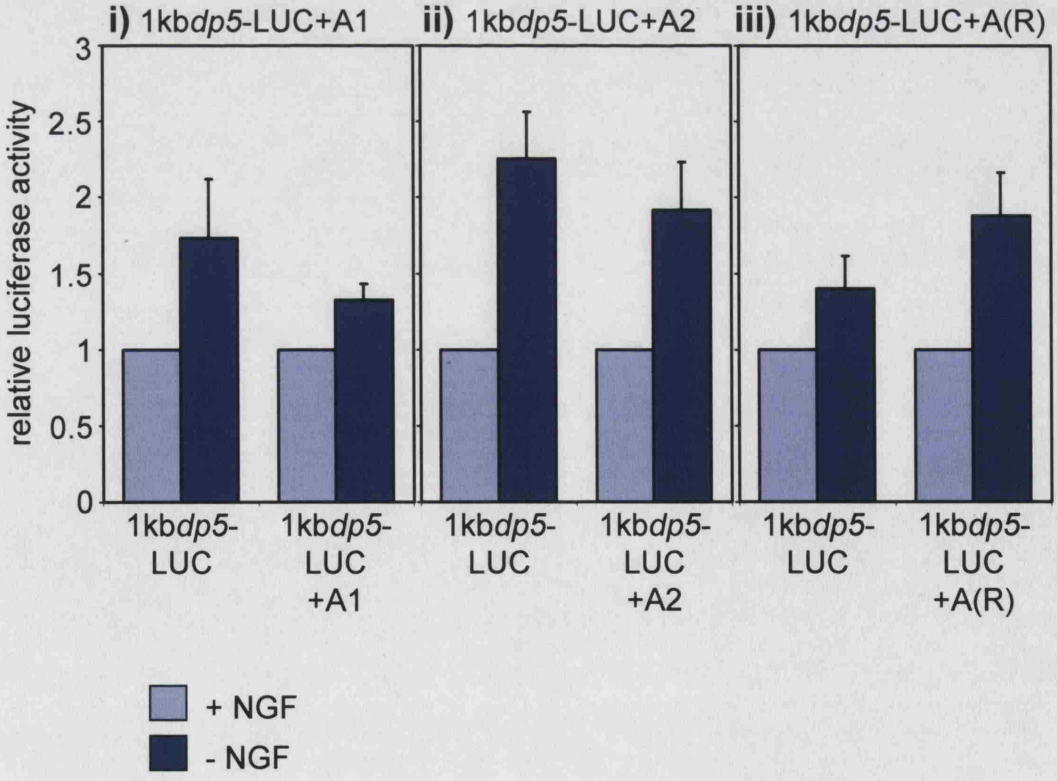
ii) 1kb*dp5*-LUC+A2



iii) 1kb*dp5*-LUC+A(R)



B



The addition of fragment A to a construct containing 1kb of the *dp5* promoter increases luciferase induction after NGF deprivation. This could be due to sequences in Fragment A either functioning as a transcriptional enhancer or regulating *dp5* mRNA stability. To assess the mechanism by which NGF might be regulating luciferase expression in neurons microinjected with 1kb*dp5*-LUC+A(F), a reporter construct containing fragment A in the reverse orientation (1kb*dp5*-LUC+A(R), Figure 3.10Aiii) was tested by microinjection into sympathetic neurons. After NGF withdrawal, the 1kb*dp5*-LUC+A(R) construct had a slightly higher level of induction than the 1kb*dp5*-LUC control but the mean levels of induction had overlapping errors (Figure 3.10Biii). Although a small increase in the level of induction was observed, this was lower than the level measured for 1kb*dp5*-LUC+A(F). If fragment A contained a region that functions as an enhancer, a similar increase in luciferase induction would be expected when this sequence was cloned in either orientation. However, if fragment A contained sequences that regulate mRNA stability, luciferase induction with the fragment in the reverse orientation would be comparable to that observed when the fragment was not present at all. This preliminary result suggests that fragment A contains sequences that regulate RNA stability rather than the rate of transcription initiation, as would be the case for a 3' enhancer. However further experiments are required to substantiate this hypothesis.

3.3 Discussion

In this chapter I have described the structure of the rat *dp5* gene and have identified regions that are involved in the induction of *dp5* expression in response to NGF deprivation in sympathetic neurons. The strategy used was to build luciferase reporter constructs containing different regions of the gene and then microinject these into sympathetic neurons and measure luciferase expression after NGF withdrawal. The results demonstrate that the *dp5* promoter, intron and 3'UTR all contain elements that regulate *dp5* expression in response to NGF deprivation in this system.

Despite sympathetic neurons being a useful cell culture model for studying neuronal apoptosis, the lack of an efficient transfection method has been a major limitation. Techniques using calcium phosphate precipitation, electroporation,

lipofection and polyethyleneimine-mediated transfection do not result in high enough levels of reporter expression (Pajak *et al.*, 2003) and although recombinant adenoviruses have been successfully used to transfer DNA, problems can arise when using long DNA sequences. The direct microinjection of reporter constructs into the nuclei of sympathetic neurons has successfully overcome some of these problems without dramatically affecting neuron viability (Garcia *et al.*, 1992;Pajak *et al.*, 2003), but this technique depends upon a reliable and sensitive method for detecting and quantifying changes in reporter gene expression in just a small number of cells. Luciferase expressing constructs are a suitable reporter system, allowing reporter gene expression to be measured by either visualisation of stained microinjected cells or quantification of luciferase activity, both of which were successfully used to study the transcriptional regulation of the BH3-only subfamily member *bim* (Gilley *et al.*, 2003). Assaying microinjected cells co-injected with a protein marker by using immunofluorescence is an established technique for determining reporter gene expression but this method is only semi-quantitative since neurons are only scored as to whether they express luciferase protein or not. The degree of luciferase expression cannot be measured and this can vary considerably between individual cells. This problem was overcome by adapting the dual luciferase assay to measure changes in luciferase expression in microinjected sympathetic neurons after NGF withdrawal. Co-injecting a *Renilla* luciferase construct with a constitutively active promoter allows normalisation of firefly luciferase activity, correcting for variation in the amount of injection mix injected and differences in the number of cells injected. My results indicate that these two methods of measuring luciferase expression are comparable since both immunofluorescence and the dual luciferase assay gave the same change in the pattern of expression with the same pair of *dp5* reporter constructs. The latter has the advantage of being a fully quantitative assay with a greater sensitivity for increases in luciferase expression, reflected by luciferase induction being detected where previously only a negligible increase was seen after NGF withdrawal when using immunofluorescence. The dual luciferase assay is also much less time consuming and requires fewer cells to be injected for each construct. It has been reported that the promoter activity of a luciferase reporter plasmid in a single

injected neuron can be detected (Pajak *et al.*, 2003), indicating the sensitivity of this assay and supporting its use in the majority of experiments carried out for this project.

Microinjection is a useful technique for allowing the transfer of foreign DNA into sympathetic neurons but because this is a technically difficult and time consuming process the number of reporter constructs that could be injected and tested together was limited. Therefore it was important to ensure that a control was used throughout and in most cases 1kb*dp5*-LUC was used as a standard. The luciferase induction measured for other constructs after NGF withdrawal could be compared to this allowing any differences due to the addition of other regions of the *dp5* gene to be determined. It was important to ensure that variation within each experiment was kept to a minimum so that different constructs and the effect of the presence and absence of NGF could be fairly compared. To limit this variation, cells for each individual experiment were from the same neuron preparation and were microinjected on the same day at the same time. Other factors that were kept constant for each repeat included the time between microinjection and NGF deprivation and the use of the same aliquot from the dual luciferase assay kit to measure luciferase activity for each experiment. It was also important that different constructs were microinjected at near equimolar concentrations because some constructs differed dramatically in size and so by adjusting concentrations to take this into account, it was more likely that a similar copy number of each construct would be injected.

There was some variation that occurred between repeat experiments reflected by the spread of induction for 1kb*dp5*-LUC after NGF withdrawal when comparing all the results obtained for this construct. The sequence 1 kb immediately upstream of the *dp5* transcriptional start site does appear to contain an element that causes higher levels of *dp5* expression after NGF withdrawal, demonstrated by the increase in luciferase expression in the absence of NGF. Results from the experiments testing a reporter construct containing a shorter region of the promoter (150bp*dp5*-LUC) suggested that the promoter regulatory element is not contained within the 150 bp at the 3' end of the 1 kb promoter sequence or that this sequence alone is not sufficient for the increase in expression after NGF deprivation to occur. It appears that the time after NGF deprivation at which the luciferase assay was performed could alter the level of

luciferase induction measured, indicated by the changes in the induction levels between 16, 20 and 24 hours when either 1kb*dp5*-LUC or 1kb*dp5*-LUC+A(F) were injected. These results suggest that the pattern of expression with these constructs remained constant but indicate the importance of keeping the period of NGF deprivation constant between repeat experiments. It is possible that varying the time of NGF withdrawal could account for the differences in induction seen across the 40 experiments using 1kb*dp5*-LUC. Other sources of variation between repeat experiments could occur through using neurons from different preparations and by microinjecting cells after a different number of days *in vitro*.

My results demonstrate that although a reporter construct containing 1 kb of *dp5* promoter sequence did show increased levels of luciferase expression following NGF deprivation, this response was increased by the addition of sequences from either the *dp5* intron or 3'UTR. This implies that there are also sequences within these regions that increase the induction of *dp5* expression after NGF withdrawal. The presence of all three of the *dp5* regions in a reporter construct, 1kb*dp5*-LUC+ALL, resulted in the greatest induction after removal of NGF from the medium, again suggesting that each of these regions contain elements that regulate expression in response to NGF withdrawal, having the largest effect when present together. In addition to aiding the identification of important sequences for regulating the response to NGF deprivation, obtaining a *dp5* luciferase reporter construct that showed a 4.81 fold increase in expression after NGF withdrawal is also useful for studying other aspects of *dp5* regulation. In having a construct with a relatively large increase in expression after NGF withdrawal, any decreases in induction, for example due to mutating potential regulatory sequences, will be more readily detectable.

Due to its large size (~18.9 kb) it was not feasible to insert the entire intron into a reporter construct, therefore the intron DNA sequence was examined to identify any regions with high levels of conservation between the rat, mouse and human sequences. Finding a highly conserved region in a sequence of non-coding DNA would imply that this region played a functionally significant role and was likely to be involved in gene regulation (Hardison, 2000;Lenhard *et al.*, 2003;Pennacchio and Rubin, 2001). This led to the identification of a ~400 bp conserved region in the *dp5* intron and the addition of

this relatively short sequence from the intron into a construct containing the *dp5* 1 kb promoter region increased the induction of luciferase after NGF deprivation, compared to the promoter alone. The intron sequence was inserted downstream of the SV40 poly-A transcription termination signal, rather than within the 3'UTR as in the *dp5* gene. Therefore if fragment I altered induction this would be due to a sequence within this region acting as an enhancer, since enhancers are defined as functioning independently of location and orientation. Enhancers can also act independently of their promoter and are able to increase the transcriptional activity of other non-specific promoters. The fact that fragment I did not appear to increase transcriptional activity after NGF withdrawal when located upstream of the SV40 promoter indicates that this fragment was not acting as an enhancer for the SV40 promoter. This implies that the intron enhancer functions in a promoter-specific manner in which the presence of the enhancer caused an increase in promoter activity only when in the context of the *dp5* promoter and does not alter the response to NGF deprivation when in the context of a heterologous promoter. In a similar manner, the intronic regulatory elements of the murine complement receptor type 2 (CD21) gene show promoter specificity as the CD21 intron 1 confers cell-specific expression to the CD21 promoter but this cell-specific control is lost when the intron is inserted next to the SV40 promoter (Zabel *et al.*, 2000). This type of context-specific regulation suggests that there are specific elements in the promoter that are required for intronic sequences to act as regulatory elements and confer cell specific promoter activity or activity in reponse to external stimuli, possibly requiring specific sequence or protein associations between the intron and promoter regions. The fragment I enhancer could be further characterised by inserting this sequence in the reverse (3' to 5') orientation in the context of the *dp5* promoter to confirm that this sequence is functioning as an enhancer. Reporter constructs could also be made that contain this intron fragment with different lengths and regions of the *dp5* promoter to identify the sequence within the promoter that is required for enhancer activity. For example, the intron could be inserted into 150bp*dp5*-LUC, which contains 150 bp of the *dp5* promoter, as despite this region having a reduced response to NGF deprivation it could still contain a sequence that responded in the presence of fragment I to cause an enhanced increase in promoter activity after NGF deprivation.

The presence of the *dp5* 3'UTR in addition to the 1 kb of sequence from the *dp5* promoter region also increased the induction of luciferase after NGF withdrawal, suggesting that this region also contains an element that regulates the expression of *dp5* after NGF deprivation. The 3'UTR is relatively large and a region that responded to NGF withdrawal was localised to fragment A, a smaller 645 bp sequence that includes the most highly conserved region of the 3'UTR when comparing the rat, mouse and human sequences. The effect of this sequence was tested using immunofluorescence and the dual luciferase assay to measure luciferase induction and these results, combined with those obtained for the timecourse carried out, suggested that the presence of this region of 3'UTR caused an increase in luciferase expression after NGF withdrawal. Furthermore, this increase was only observed when the 3'UTR sequence from either side of the intron was present because constructs containing the sequence from only exon 1 or only exon 2 did not show any increase in the level of induction after NGF deprivation compared to when both of these regions were present together. This indicates that the potential DRE in exon 2, which was identified as an element that regulates *hrk* expression in response to IL-3 withdrawal in IL-3-dependent haematopoietic progenitor cells (Sanz *et al.*, 2001), does not act as a regulatory element in response to NGF deprivation in sympathetic neurons. To obtain the levels of induction previously observed for fragment A, sequences from either side of intron, i.e. both fragments A1 and A2, are required. It is possible that the remainder of the 3'UTR sequence could also contain some regulatory regions that respond to NGF withdrawal and this could be tested in future microinjection experiments by making reporter constructs that contain other regions from further downstream in the 3'UTR. This would help to establish whether all the regulatory regions from the 3'UTR have been identified.

It appears that fragment A contains an NGF-responsive element but because this element was located downstream of the ORF, in the same context as in *dp5*, it cannot be established whether this region functions as an enhancer. To determine this, a construct was made containing this fragment in the reverse (3' to 5') orientation. If fragment A did contain an enhancer element, a similar level of luciferase induction would be expected in the forward and reverse orientations. Although an increase in luciferase induction was observed after NGF deprivation, the increase was only marginally greater than that

obtained with 1kb*dp5*-LUC (p=0.473). It is therefore possible that this sequence does not contain an enhancer element but functions to increase *dp5* levels by stabilising mRNA, consequently reducing mRNA degradation. Examination of the sequence did not identify any obvious ARE but this hypothesis could be tested further in future experiments. Reversing fragment A appeared to decrease the level of luciferase expression after NGF deprivation and a similar experiment could be performed in which this fragment was inserted upstream of a promoter to establish if it functions as enhancer. The construct could be inserted either upstream of a heterologous promoter, such as the SV40 promoter, or upstream of the *dp5* promoter if the sequence only functions in a context-specific manner. The role of mRNA stability in regulating *dp5* levels in response to NGF deprivation could also be investigated by examining whether *dp5* mRNA becomes more stable in the absence of NGF. This could be done by inhibiting transcription, through use of a chemical inhibitor such as actinomycin D, and then measuring mRNA levels after various times in the presence or absence of NGF by RT-PCR or real time PCR. If RNA stability is involved in *dp5* regulation, levels of *dp5* mRNA would not decrease as rapidly in the absence of NGF compared to when NGF was present, due to an increase in mRNA stability after NGF withdrawal. This result would indicate whether this mechanism of regulation was involved and combined with the results from the further microinjection experiments would suggest whether the sequence within fragment A could affect mRNA stability.

In the next chapter I will present the results of experiments in which I used some of the NGF-responsive *dp5* reporter constructs described in this chapter to investigate the role of different signalling pathways in *dp5* regulation in sympathetic neurons.

4 Investigation of the signalling pathways involved in the regulation of *dp5*

4.1 Introduction

Apoptosis during the development of the nervous system is a tightly controlled process that involves the interaction of multiple signalling pathways which regulate the expression and activity of the proteins involved in cell death. In the sympathetic neuron model, the withdrawal of NGF from the growth medium dramatically alters the activity of signalling pathways resulting in the induction or repression of downstream target genes.

NGF promotes neurite outgrowth and neuronal survival by binding to the tyrosine kinase receptor TrkA on the surface of neurons. The binding of NGF to these receptors stimulates downstream protein kinase activity by triggering the autophosphoryation of tyrosine residues within the TrkA cytoplasmic tail, leading to the activation of the signalling pathways that coordinate survival including the phosphoinositide 3-kinase (PI3-K) and mitogen-activated protein kinase (MAPK) pathways (Freeman *et al.*, 2004;Sofroniew *et al.*, 2001). After NGF withdrawal, the TrkA receptor becomes dephosphorylated resulting in the alteration of intracellular signalling pathways, including a decrease in PI3-K and MAPK activity and an increase in c-Jun N-terminal kinase (JNK) activity (Chang *et al.*, 2002). This change in activity affects the expression of downstream targets, including genes involved in the regulation of apoptosis.

The role of the PI3-K pathway as a regulator of neuronal survival was first established during experiments in which the treatment of PC12 cells with the specific PI3-K pathway inhibitors wortmannin or LY294002 prevented cell survival in the presence of NGF (Yao and Cooper, 1995). This pathway has also been shown to be important for the NGF-dependent survival of sympathetic neurons (Bartlett *et al.*, 1997). The PI3-K pathway is known to regulate the expression of the BH3-only protein BIM since the inhibitor LY294002 increases *bim* RNA and BIM protein levels in the presence

of NGF in sympathetic neurons, indicating that this pathway normally inhibits *bim* expression (Gilley *et al.*, 2003).

In contrast to PI3-kinase, there is a marked increase in JNK activity in sympathetic neurons following NGF deprivation (Eilers *et al.*, 1998;Virdee *et al.*, 1997). The JNK pathway activates the transcription factor c-Jun, a member of the AP-1 family, and c-Jun levels, as well as c-Jun N-terminal phosphorylation, increase in sympathetic neurons after NGF withdrawal. c-Jun plays a key role in apoptosis as demonstrated by the protective effect of expressing a c-Jun dominant negative mutant (dn-Jun) or by injecting a neutralising antibody specific for c-Jun (Estus *et al.*, 1994;Ham *et al.*, 1995) or by conditional inactivation of the *c-jun* gene in sympathetic neurons (Palmada *et al.*, 2002). JNK controls c-Jun activity by phosphorylating the c-Jun transcriptional activation domain. Experiments in which the JNK-binding domain of JIP-1, which functions as a selective JNK inhibitor, was expressed in sympathetic neurons demonstrated that JNK activity is required not only for c-Jun phosphorylation but also *c-jun* promoter activation and NGF withdrawal-induced apoptosis (Eilers *et al.*, 2001;Harding *et al.*, 2001). c-Jun has been suggested to contribute to the transcriptional induction of *bim* because expression of dn-Jun reduces the level of *bim* mRNA induction by ~50% in NGF-deprived sympathetic neurons (Sanchez and Yuan, 2001;Whitfield *et al.*, 2001). The upstream JNK pathway has also been implicated in BIM regulation though use of the small molecule mixed lineage kinase (MLK) inhibitor CEP-1347, which prevents JNK activation. Like dn-Jun, this inhibitor reduces *bim* induction, decreasing the level of *bim* mRNA by 85% in sympathetic neurons deprived of NGF, and prevents NGF withdrawal-induced death (Harris and Johnson, Jr., 2001). The involvement of at least two signalling pathways in *bim* transcriptional regulation indicates the potential complexity involved in controlling the expression of the BH3-only proteins, essential for the stringent regulation of apoptosis.

The JNK pathway has also been implicated in *dp5* regulation by the use of the MLK inhibitor CEP-1347. Inhibition of the MLK/JNK pathway results in reduced *dp5* mRNA induction in both potassium-deprived cerebellar granule neurons and NGF-deprived sympathetic neurons by ~80% and ~75% respectively, suggesting that this part of *dp5* mRNA induction can be accounted for by the JNK signalling pathway (Harris

and Johnson, Jr., 2001). However, the specificity of this MLK/JNK pathway inhibitor needs to be considered since CEP-1347 has also been shown to activate the PI3-K/Akt signalling pathway (Roux *et al.*, 2002;Wang *et al.*, 2005).

In the experiments outlined in this chapter, the role of the JNK and PI3-K signalling pathways in *dp5* induction have been investigated further by the use of a MLK3 expression vector and the chemical inhibitors CEP-11004 and LY294002. The JNK pathway is activated by upstream mixed linage kinases (MLKs) and expression of an active wild type MLK3 protein is sufficient to induce an increase in phosphorylated c-Jun levels and apoptosis in sympathetic neurons. After NGF withdrawal, increases in phosphorylated c-Jun and apoptosis can be prevented by the overexpression of a kinase dead MLK3 mutant (Mota *et al.*, 2001), making these expression vectors a useful tool for studying regulation by the MLK/JNK pathway. This pathway can also be studied using the MLK inhibitor CEP-11004, a compound that is closely related to the small molecule inhibitor CEP-1347 (Murakata *et al.*, 2002). These compounds target the JNK pathway through the selective inhibition of upstream MLKs and CEP-1347 has been shown to completely block JNK activation and protect sympathetic neurons from NGF withdrawal-induced apoptosis at 200 nM (Maroney *et al.*, 1999; Harris *et al.*, 2002). Work carried out in our laboratory by Dr. Jonathan Gilley has indicated that the related compound CEP-11004 has a similar effect in sympathetic neurons because treatment of these cells with this inhibitor decreases the level of c-Jun induction after NGF withdrawal (Figure 4.1), suggesting a reduction in MLK/JNK pathway activity. The induction of BIM, a known target of the JNK pathway, is also reduced in sympathetic neurons treated with CEP-11004 after NGF deprivation (Figure 4.1), indicating that this compound has a similar effect to CEP-1347. The chemical inhibitor LY294002 specifically inhibits PI3-K activity and this compound has been shown to effectively inhibit PI3-K in sympathetic neurons, preventing cell survival in the presence of NGF (Bartlett *et al.*, 1997) and making this a suitable inhibitor to study the role of the PI3-K/Akt pathway.

In some of the experiments described in this chapter, the PC12 cell line was used as a neuronal cell model. These cells are derived from a rat adrenal pheochromocytoma and in the presence of serum will divide and resemble precursors of adrenal chromaffin

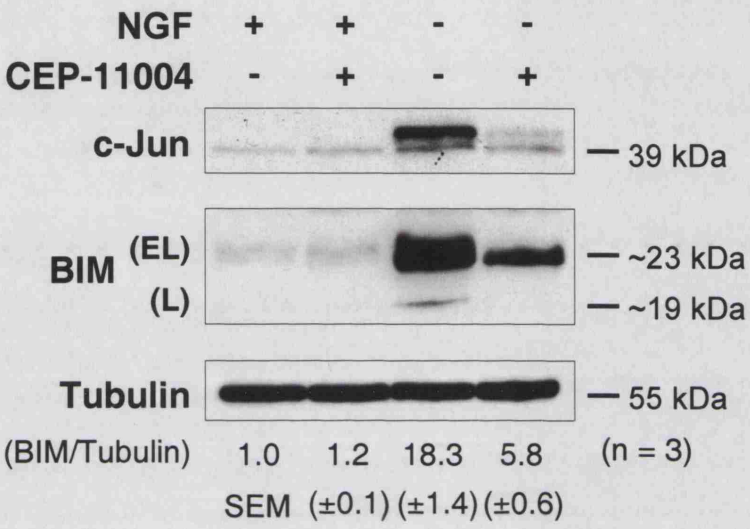


Figure 4.1 Effect of the MLK inhibitor CEP-11004 on the expression of the c-Jun and BIM proteins in sympathetic neurons

These experiments were performed by Dr. Jonathan Gilley. Sympathetic neurons were treated with DMSO (-) or CEP-11004 at 400 nM (+) in the presence or absence of NGF for ~16 hours before protein was extracted and the expression of c-Jun and BIM examined by immunoblotting. Relative protein levels were quantified by densitometry and normalised to the level of tubulin. The mean of 3 experiments ± the SEM is shown beneath representative immunoblots.

cells (Figure 4.2A). On the addition of NGF, these cells differentiate and develop a neuron-like morphology (Figure 4.2B). After one week in the presence of NGF, these cells have stopped proliferating and show extensive neurite outgrowth, resembling sympathetic neurons in primary culture (Figure 4.2C; Francois *et al.*, 2001;Greene and Tischler, 1976;Lambeng *et al.*, 2003;Vaudry *et al.*, 2002). The PC6.3 subline of PC12 cells provides a particularly good model for neuronal cell death because 90% of these cells undergo apoptosis after the removal of NGF (Figure 4.2D). This death in response to NGF withdrawal can be blocked by actinomycin D and cycloheximide, inhibitors of RNA and protein synthesis respectively, making PC6.3 cells a suitable model for neuronal apoptosis (Pittman *et al.*, 1993). These cells are useful because they can be efficiently transfected using a lipofection technique, overcoming some of the limitations of using primary sympathetic neurons.

This chapter presents the results of experiments in which different *dp5* luciferase reporter constructs containing major regions of the *dp5* gene were tested by microinjection into sympathetic neurons, which were then treated with a chemical inhibitor, or alternatively the cells were co-injected with a MLK3 expression vector. Some repeated microinjection experiments were done in collaboration with my supervisor, Dr. Jonathan Ham.

4.2 Results

4.2.1 Wild type and kinase dead MLK3 expression vectors behave as expected in transiently transfected PC6.3 cells

Before being used in microinjection experiments, a human wild type activated MLK3 expression vector and a kinase dead MLK3 expression vector (see Section 2.1.3) were transiently transfected into neuronally differentiated PC6.3 cells to test whether these constructs functioned in the expected way. Firefly luciferase reporter constructs containing either the SV40 promoter (pGL3-P), the human *c-jun* promoter (*c-jun*-LUC) or the *c-jun* promoter with mutated jun1 and jun2 TRE sites (j1j2-LUC; see Section 2.1.3) were co-transfected with either the empty vector pcDNA3, the wild type MLK3 construct or the kinase dead MLK3 vector as well as a *Renilla* luciferase reporter

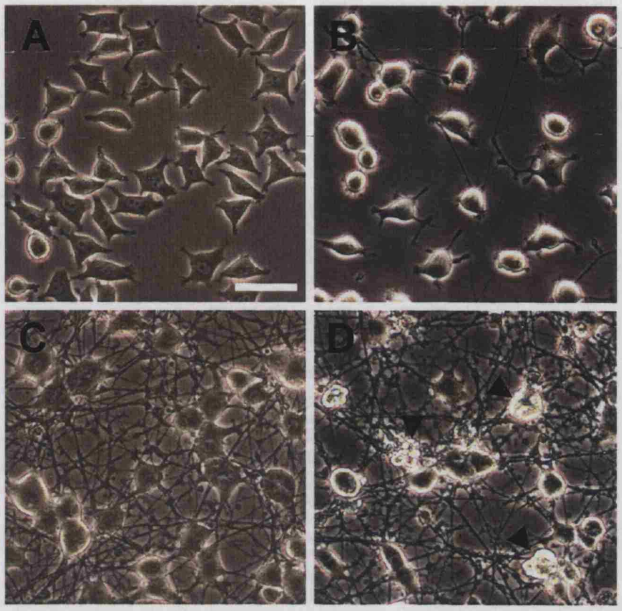


Figure 4.2 Morphology of naïve and neuronally-differentiated PC6.3 cells

Naïve PC6.3 cells were maintained *in vitro* in the absence of NGF (**A**). Following the addition of NGF these cells develop a neuron-like morphology (**B** and **C**, 3 and 7 days after the addition of NGF respectively). If NGF is removed from the medium, neuronally-differentiated PC6.3 cells undergo apoptosis with the characteristic blebbing morphology associated with this type of cell death (**D**, 24 hours after NGF deprivation). Apoptotic cells are indicated by the black arrowheads. The scale bar represents 40 μm.

construct (pRL-TK). Luciferase activity was determined 24 hours after lipofection using the dual luciferase assay and firefly luciferase activity was calculated relative to *Renilla* luciferase activity to control for variations in the transfection efficiency. The luciferase activity for each reporter construct with the different expression vectors was calculated relative to the activity observed with the co-transfected pcDNA3 control (Figure 4.3). The SV40 promoter is not activated by the MLK/JNK pathway and, as predicted, neither the wild type nor the kinase dead MLK3 expression vectors altered the activity of pGL3-P. In contrast to this, the *c-jun* promoter, a known target of the MLK/JNK pathway, was activated approximately 3 fold by the co-transfected wild type MLK3 vector whereas the presence of the kinase dead MLK3 expression vector resulted in only a 1.3 fold increase in promoter activity, a significantly reduced level compared to wild type MLK3 ($p=<0.01$). The j1j2-LUC construct containing the mutated *c-jun* promoter showed highly reduced relative levels of activation when co-transfected with kinase active MLK3 compared to the wild type promoter and, in addition to this, the kinase dead MLK3 construct did not appear to decrease this level of activation in the same manner as with the wild type promoter. In these experiments, all of the reporter constructs responded in a similar manner at both of the concentrations of co-transfected expression vector tested. These results suggest that the MLK3 expression vectors behave as expected because the wild type construct increases the activity of a known target of the MLK/JNK pathway (*c-jun* promoter), whereas the kinase dead form has a dramatically reduced effect on the same target. Both expression vectors have a similar minimal effect on a mutant promoter (j1j2) that has a reduced response to targets of the MLK/JNK pathway (Angel *et al.*, 1988). This indicates that these expression vectors could be used to determine the effect of altering the activity of the MLK/JNK pathway on different *dp5* reporter constructs.

4.2.2 Overexpression of wild type MLK3 activates *dp5* reporter constructs containing specific regions of the *dp5* gene

As reported in chapter 3, 1 kb of the *dp5* promoter sequence from directly upstream of the transcriptional start site, the conserved region of the intron and the 3'UTR appear to

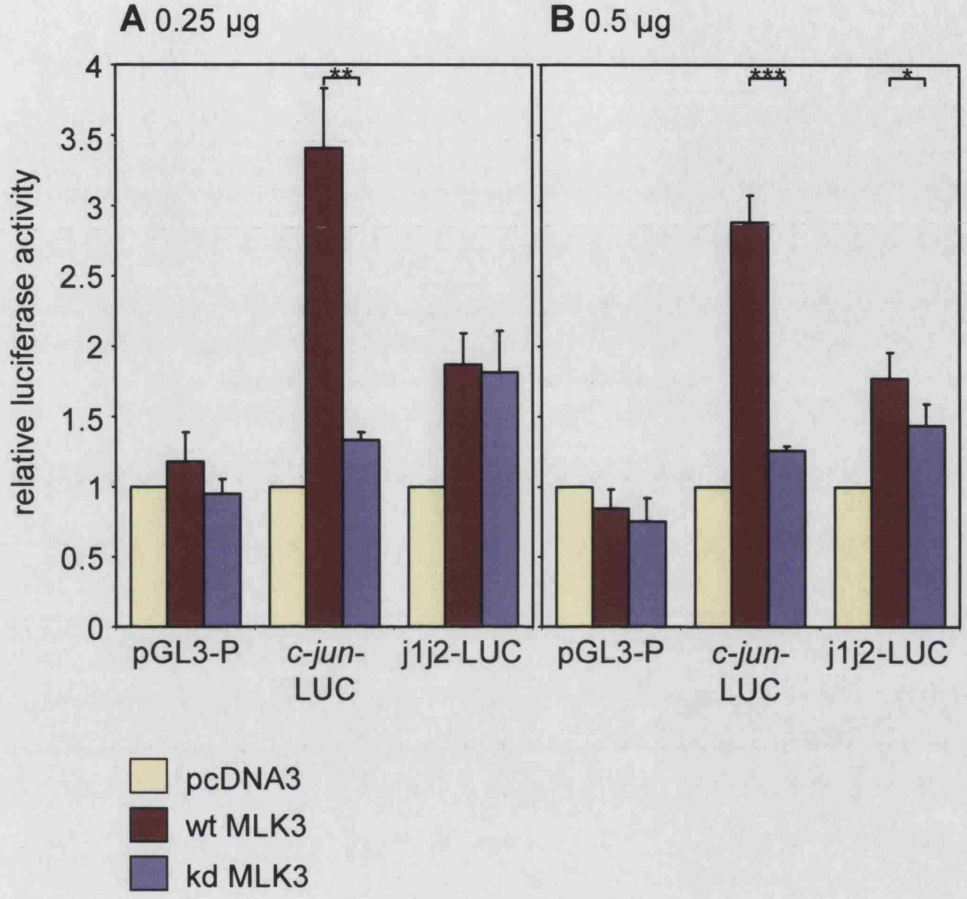


Figure 4.3 A wild type MLK3 expression vector activates a promoter known to be regulated by the MLK/JNK pathway in transiently transfected neuronally differentiated PC6.3 cells

Neuronally differentiated PC6.3 cells were transiently transfected with either 0.2 µg of pGL3-P (containing the SV40 promoter), *c-jun*-LUC or j1j2-LUC (containing the *c-jun* promoter with mutated jun1 and jun2 TRE sites) together with 0.05 µg pRL-TK. Also, either **A)** 0.25 µg or **B)** 0.5 µg of a control vector (pcDNA3), a wild type (wt) MLK3 expression vector or a kinase dead (kd) MLK3 expression vector was co-transfected. Luciferase activity was measured after 24 hours using a dual luciferase assay. The mean of 3 duplicates is shown ± the SEM (** $P<0.01$, *** $P<0.001$, *t*-test).

contain elements that regulate *dp5* expression in response to NGF deprivation. To determine whether these regions contain sequences that respond to the MLK/JNK pathway, the effect of activating this pathway on the constructs 1kb*dp5*-LUC, 1kb*dp5*-LUC+I(F) and 1kb*dp5*-LUC+3'UTR was tested by using the wild type MLK3 expression vector described above. Co-injection experiments were performed in which the three *dp5* constructs, as well as *c-jun*-LUC, were microinjected into sympathetic neurons together with either the wild type MLK3 or kinase-dead MLK3 expression vector. A *Renilla* luciferase construct (pRL-TK) was also microinjected. Following injection, the neurons were maintained in medium containing NGF for 16 to 20 hours and after this time luciferase activity was determined by performing a dual luciferase assay. Luciferase activity in the cells co-injected with the wild type MLK3 construct was calculated relative to those injected with the kinase dead MLK3 construct (Figure 4.4). The *c-jun*-LUC reporter construct was again strongly activated by the wild type MLK3 expression vector compared to the kinase dead control (7.2 fold increase). The *dp5* reporter constructs 1kb*dp5*-LUC and 1kb*dp5*-LUC+I(F) also appeared to have increased activity in the presence of active MLK3 as the relative luciferase activity increased 1.8 fold and 2.1 fold respectively when co-injected with wild type MLK3 compared to the kinase dead expression vector. In contrast to this, the reporter construct that contained the 3'UTR in addition to the promoter region showed no detectable difference in activity with either kinase active or kinase dead forms of MLK3. These results suggest that the *dp5* promoter region contains an element that increases *dp5* promoter activity after the activation of the MLK/JNK pathway, whereas an element in the 3'UTR appears to prevent this activation. The contribution of any additional sequences in the intron that are a target of MLK/JNK signalling cannot be detected in this experiment because although 1kb*dp5*-LUC+I(F) is activated by wild type MLK3 this construct also contains the 1 kb of promoter sequence that on its own can respond to MLK3. Although the activity of both 1kb*dp5*-LUC and 1kb*dp5*-LUC+I(F) increases in the presence of active MLK3, the results with the construct containing the promoter alone did show some variability reflected by the standard error of the mean. It was therefore important to further investigate the role of the MLK/JNK pathway by examining the effect of inhibiting this pathway.

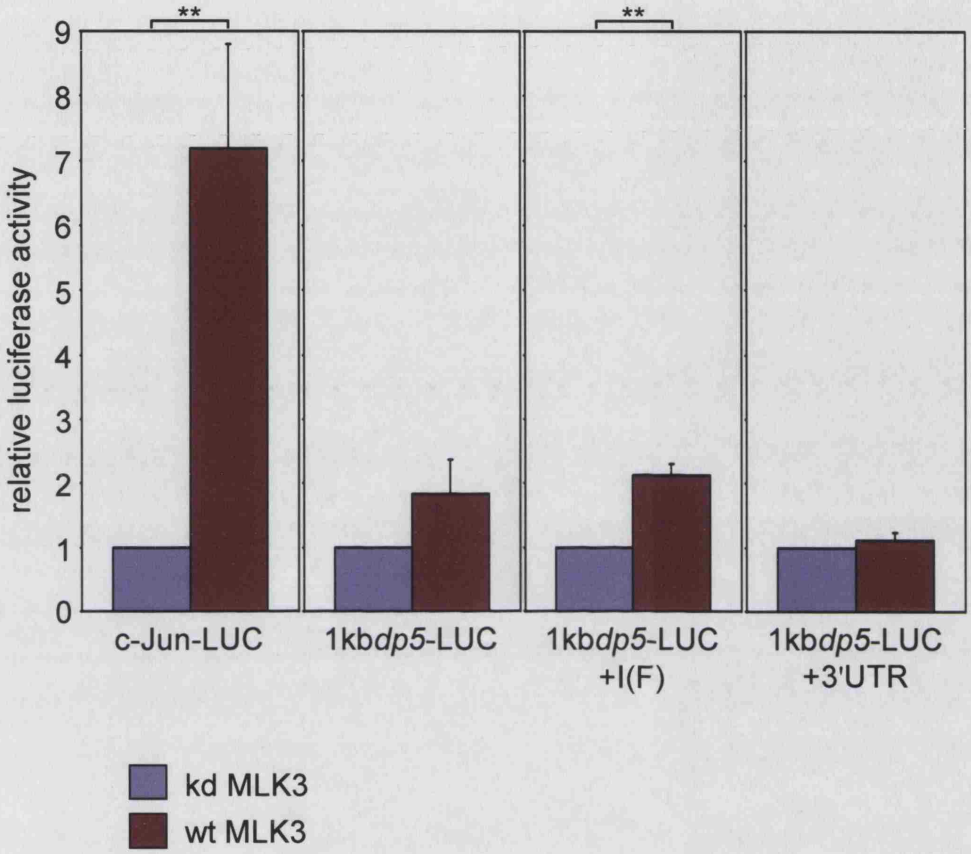


Figure 4.4 Overexpression of wild type MLK3 activates *dp5* constructs containing the *dp5* promoter alone or with a sequence from the intron, relative to a kinase dead MLK3 expresion vector in sympathetic neurons

Sympathetic neurons were microinjected with either *c-jun*-LUC, 1kb*dp5*-LUC, 1kb-*dp5*-LUC+I(F) or 1kb*dp5*-LUC+3'UTR [0.01 μg/μl] as well as pRL-TK [0.005 μg/μl]. Expression vectors for wild type (wt) MLK3 or kinase dead (kd) MLK3 [0.1 μg/μl] were also co-injected as indicated. Luciferase activity was determined using a dual luciferase assay and the luciferase activity in the presence of wt MLK3 was calculated relative to the level with kd MLK3, which was set at 1. The mean of at least 5 experiments ± the SEM is shown (** $P<0.01$, *t*-test).

4.2.3 The MLK inhibitor CEP-11004 reduces *dp5* expression after NGF withdrawal

The compound CEP-11004 has been used to inhibit MLK activity in our laboratory and it was necessary to test whether this inhibitor had a similar effect on *dp5* mRNA levels after NGF deprivation as the related compound CEP-1347. Sympathetic neurons were cultured *in vitro* for 5 to 7 days and then maintained in the presence or absence of NGF and either treated with DMSO or CEP-11004 for 16 to 20 hours. After this time, RNA was extracted from the neurons and reverse transcription PCR (RT-PCR) was performed to determine the level of *dp5* mRNA under each condition. Neurofilament (*nf-m*) mRNA levels were also measured to act as a control for the total amount of mRNA present. After quantification, the adjusted level of *dp5* expression for each condition (*dp5* expression ÷ *nf-m* expression) was calculated relative to the level in cells treated with DMSO in the presence of NGF. Figure 4.5A shows a representative experiment in which *dp5* levels increased following NGF deprivation but this increase was dramatically reduced in the presence of the inhibitor CEP-11004. This is more apparent following quantification. The 4.3 fold induction of *dp5* after NGF deprivation in control DMSO conditions is significantly reduced to a 2.3 fold induction in the presence of CEP-11004 (Figure 4.5B, $p<0.05$). These results indicate that, like CEP-1347, CEP-11004 reduces the induction of *dp5* after NGF withdrawal.

4.2.4 CEP-11004 reduces the activity of *dp5* reporter constructs after NGF deprivation

The effect of activating the MLK/JNK pathway in the presence of NGF on *dp5* reporter constructs had been tested and to further examine the role of this pathway, the inhibitor CEP-11004 was used to determine the response after inhibiting MLK activity in conditions where it is normally active i.e. after NGF withdrawal. Sympathetic neurons were microinjected with either 1kb*dp5*-LUC, 1kb*dp5*-LUC+I(F) or 1kb*dp5*-LUC+3'UTR, in addition to pRL-TK, and then maintained in the presence or absence of NGF with either DMSO or CEP-11004 for 16 to 20 hours. Luciferase activity was calculated relative to the level in the presence of NGF and DMSO (Figure 4.6). In

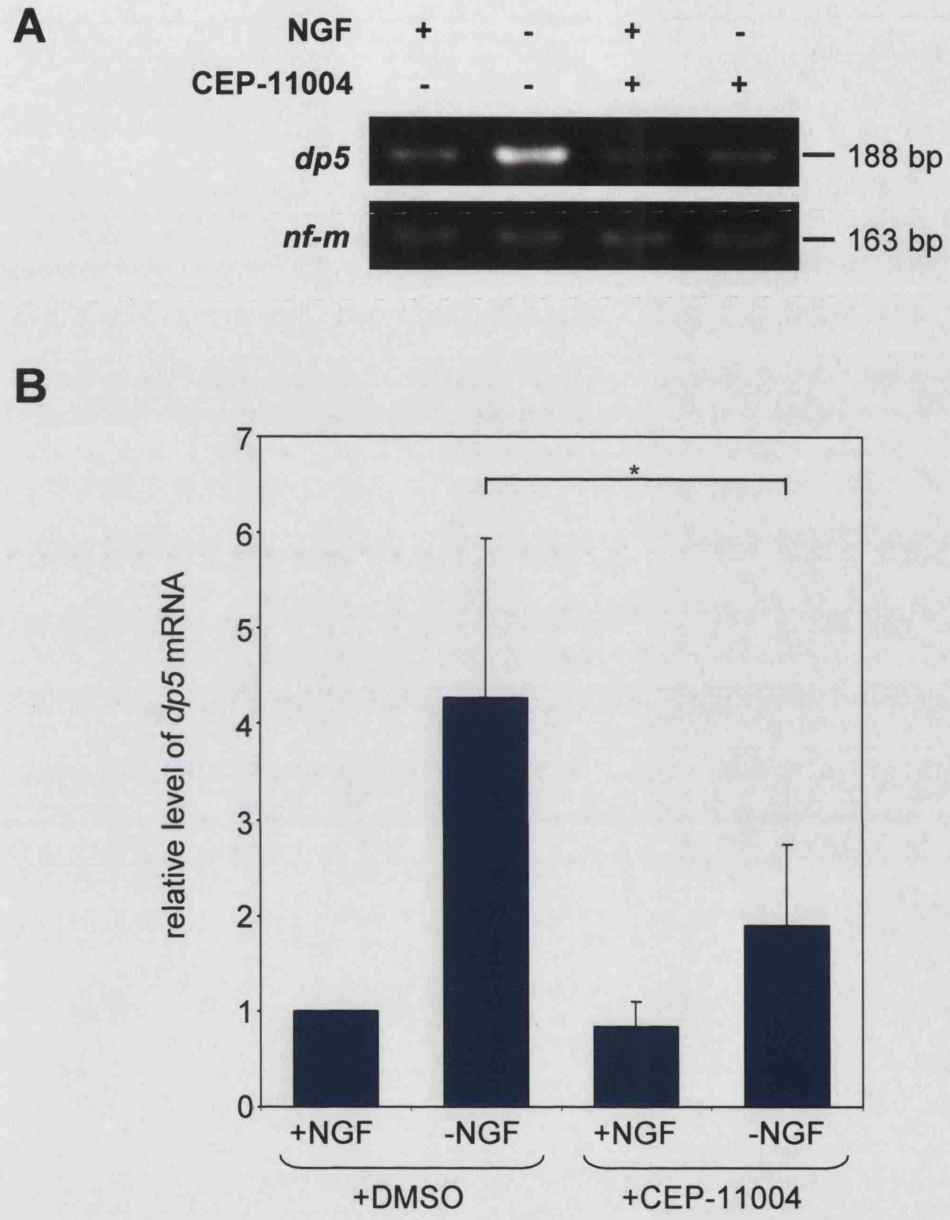


Figure 4.5 Effect of the MLK inhibitor CEP-11004 on *dp5* expression in sympathetic neurons

Sympathetic neurons were maintained in the presence or absence of NGF and were treated with either DMSO or the MLK inhibitor CEP-11004 at 400 nM for 16 hours. RNA was extracted and RT-PCR analysis carried out using *dp5* and neurofilament (*nf-m*) specific primers (**A**). The *dp5* level in each condition was calculated relative to the level in cells treated with NGF and DMSO, which was set as 1 (**B**). The mean of at least 4 experiments is shown ± SEM (* $P<0.05$, *t*-test).

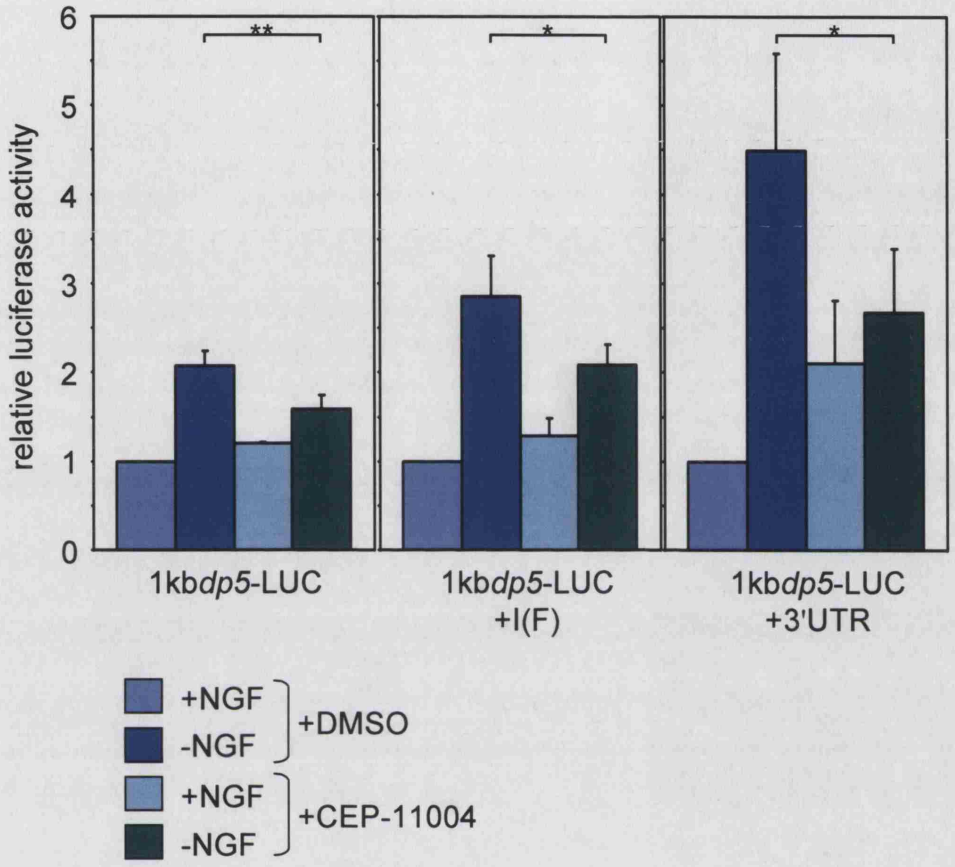


Figure 4.6 The MLK inhibitor CEP-11004 reduces the induction of *dp5* reporter constructs after NGF withdrawal in sympathetic neurons

Sympathetic neurons were microinjected with either 1kb*dp5*-LUC [0.005 μg/μl], 1kb-*dp5*-LUC+I(F) [0.005 μg/μl] or 1kb*dp5*-LUC+3'UTR [0.015 μg/μl] as well as pRL-TK [0.005 μg/μl]. Cells were then maintained in the presence or absence of NGF and were treated with either DMSO or 400 nM of the MLK inhibitor CEP-11004 for 16 hours. Luciferase activity was measured using a dual luciferase assay and the luciferase activity was calculated relative to the level +NGF treated with DMSO, which was set as 1. The mean of at least 4 experiments is shown ± the SEM (* $P<0.05$, ** $P<0.01$, *t*-test).

control cells treated with DMSO, for each of the constructs tested there was a clear increase in luciferase activity after NGF deprivation as seen previously (see Chapter 3). Although slight increases in luciferase activity were measured following NGF withdrawal for neurons treated with CEP-11004, these inductions are reduced compared to those seen after DMSO treatment. Fold increases of 2.1, 2.8 and 4.5 for the constructs 1kb*dp5*-LUC, 1kb*dp5*-LUC+I(F) and 1kb*dp5*-LUC+3'UTR are reduced to inductions of 1.3, 1.6 and 1.3 respectively after CEP-11004 treatment ($p<0.05$). After NGF withdrawal, inhibiting the MLK/JNK pathway reduces the activity of *dp5* reporter constructs containing the *dp5* promoter region alone or this region with either the intron fragment or the 3'UTR, suggesting that either the promoter, or possibly all three regions, contain a downstream target of MLK/JNK signalling. The result obtained with 1kb*dp5*-LUC and 1kb*dp5*-LUC+I(F) is consistent with the response to activation of this pathway with the wild type MLK3 expression vector (see Section 4.2.2), supporting the hypothesis that the promoter region contains a MLK/JNK-responsive element. Again, it is not possible to conclude whether the intron contains any regulated sequences on the basis of these results alone. There appear to be conflicting results for 1kb*dp5*-LUC+3'UTR when activating and inhibiting the MLK/JNK pathway because inhibition with CEP-11004 suggests that the 3'UTR either does not affect the response due to the promoter region or that it also contributes to increasing *dp5* activity after MLK/JNK activation. This result indicates that further investigation of the role of the 3'UTR and the MLK/JNK pathway is required.

4.2.5 The *dp5* 3'UTR contains a target of the PI3-K pathway

In the presence of NGF the PI3-kinase pathway is normally activated in sympathetic neurons and PI3-K activity falls after the removal of NGF from the growth medium. The role of this pathway in regulating *dp5* reporter constructs was tested by examining the effect of inhibiting this pathway by use of the specific PI3-K inhibitor LY294002. The reporter constructs 1kb*dp5*-LUC, 1kb*dp5*-LUC+I(F) and 1kb*dp5*-LUC+3'UTR were microinjected into sympathetic neurons, which were then maintained in the presence of NGF and treated with either DMSO or LY294002 for 16 hours. After this time,

luciferase activity was determined and calculated relative to the activity after treatment with DMSO (Figure 4.7). The construct containing the *dp5* promoter region alone did show a small drop in activity following LY294002 treatment, neither 1kb*dp5*-LUC nor 1kb*dp5*-LUC+I(F) showed any major difference after PI3-K inhibition. However, a reporter construct containing the 3'UTR in addition to the 1 kb promoter showed significantly increased activity (2.6 fold, $p<0.05$) after treatment with LY294002, suggesting that the 3'UTR sequence contains a downstream target of the PI3-K pathway that inhibits *dp5* expression in the presence of NGF.

It appears that the *dp5* 3'UTR contains a sequence that responds to PI3-K inhibition to increase expression in the context of a luciferase reporter construct but it was essential to establish whether inhibiting PI3-K alters endogenous *dp5* levels in sympathetic neurons. This was examined by RT-PCR analysis using RNA extracted from sympathetic neurons that had been treated with DMSO or LY294002 in the presence of NGF for 16 hours. The level of *dp5* expression was adjusted for differences in total mRNA levels using neurofilament and *dp5* expression in the presence of LY294002 was calculated relative to the DMSO control. There did not appear to be a large difference in *dp5* mRNA expression after LY294002 treatment (Figure 4.8) but the large error bars representing the standard error of the mean, as well as the spread of results, indicated a large degree of variation in these results. Although the mean level of expression does not differ greatly after inhibition of PI3-K, in some of the experiments the level did appear to increase to around 1.8 fold whereas in other experiments the levels decreased, forming two clusters of results. It is therefore impossible to conclude from this whether LY294002 has an effect on the level of expression of the endogenous *dp5* gene.

4.3 Discussion

In the experiments described in this chapter I investigated the role of different signalling pathways in the regulation of *dp5* after NGF withdrawal. The effect of altering the activity of the MLK/JNK pathway and PI3-K pathway on both endogenous *dp5* levels and on the activity of reporter constructs that contain regions of the *dp5* gene that

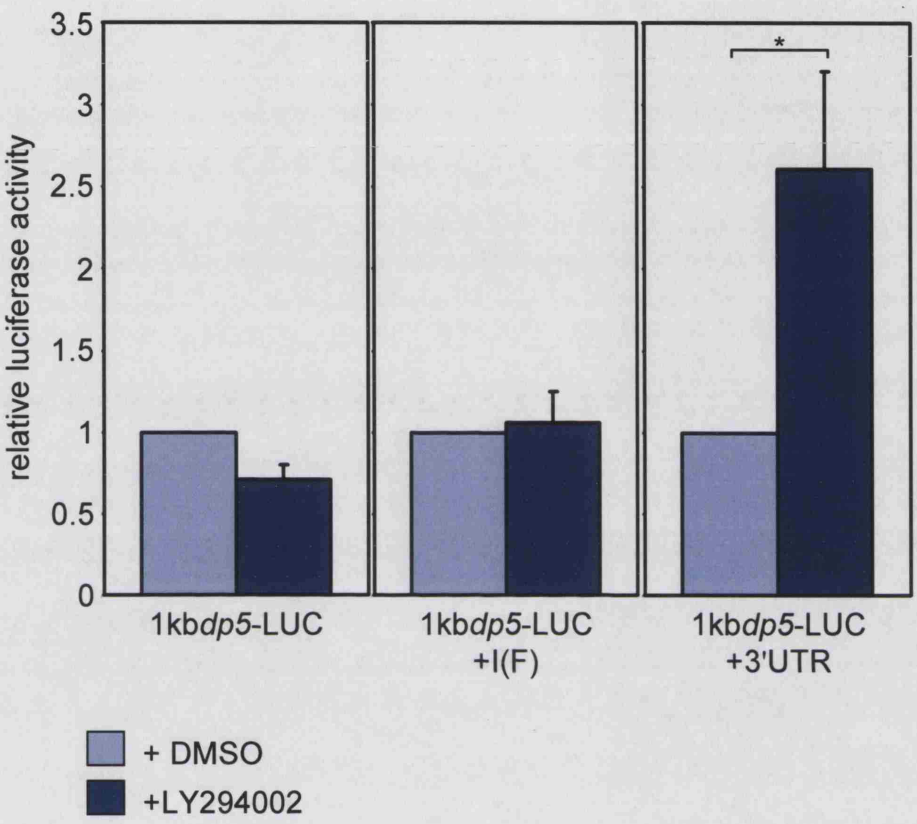


Figure 4.7 The activity of a *dp5* reporter construct containing the 3'UTR increases in the presence of the PI3-kinase inhibitor LY294002

Sympathetic neurons were microinjected with either 1kb*dp5*-LUC [0.005 μg/μl], 1kb-*dp5*-LUC+I(F) [0.005 μg/μl] or 1kb*dp5*-LUC+3'UTR [0.015 μg/μl] as well as pRL-TK [0.005 μg/μl]. Cells were then maintained in the presence of NGF and were treated with either DMSO or the PI3-kinase inhibitor LY294002 (50 μM) for 16 hours. Luciferase activity was measured using a dual luciferase assay and the luciferase activity was calculated relative to the level obtained for neurons treated with DMSO, which was set as 1. The mean of at least 4 experiments is shown ± the SEM (* $P<0.05$, *t*-test).

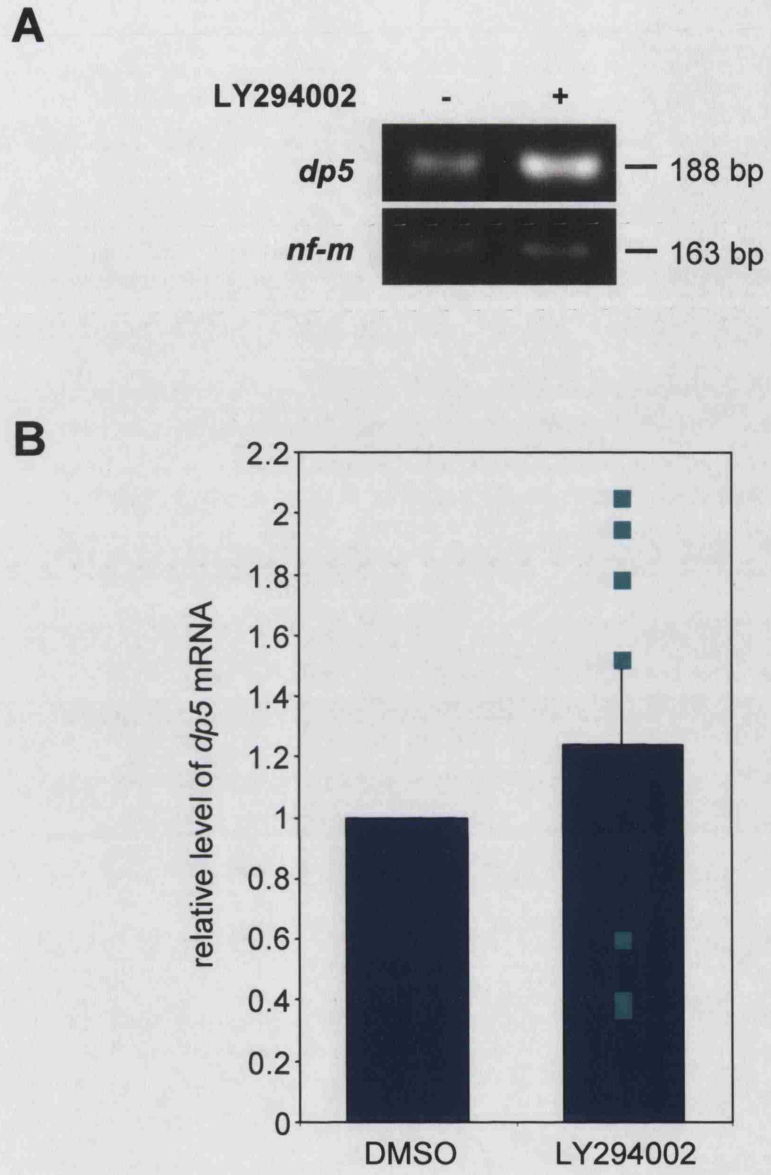


Figure 4.8 Effect of the PI3-K inhibitor LY294002 on *dp5* expression in sympathetic neurons

Sympathetic neurons were maintained in the presence NGF and were treated with either DMSO or the PI3-K inhibitor LY294002 at 50 μM for 16 hours. RNA was extracted and RT-PCR analysis carried out using *dp5* and neurofilament specific primers (**A**). The *dp5* level in the presence of LY294002 was calculated relative to the level when the cells were treated with DMSO, which was set as 1 (**B**). The mean of 7 duplicates is shown ± SEM.

respond to NGF deprivation was studied. The results obtained suggest that 1 kb of sequence upstream from the *dp5* transcriptional start site contains an element that responds to activation of the MLK/JNK pathway and that the *dp5* 3'UTR may contain a sequence that is a target of the PI3-K pathway.

In the presence of NGF, the MLK/JNK pathway is relatively inactive in sympathetic neurons but the levels of MLK and JNK activity increase following NGF deprivation. This results in c-Jun phosphorylation and apoptosis (Eilers *et al.*, 1998;Ham *et al.*, 1995). The serine/threonine kinase MLK3 is known to specifically activate the JNK and p38 pathways (Tibbles *et al.*, 1996) and microinjection of an active MLK3 expression vector has been shown to activate JNK, leading to increased c-Jun phosphorylation and the induction of apoptosis in sympathetic neurons (Mota *et al.*, 2001). MLK3 kinase activity is required for activation of the downstream JNK pathway because expression of a kinase dead dominant negative MLK3 protein, which contains a mutated ATP-binding site, blocks c-Jun phosphorylation and apoptosis in sympathetic neurons after NGF withdrawal (Mota *et al.*, 2001). Following microinjection into rat sympathetic neurons between 80% and 100% of injected cells express the wild type and kinase dead MLK3 proteins, making the MLK3 expression vectors useful tools for examining the effect of activating the MLK/JNK pathway on the regulation of *dp5* reporter constructs. The expression vectors were kindly provided by Dr. C.E. Bazenet (University College London), and following amplification, it was important to ensure that the constructs functioned as expected. This was performed by transiently transfecting PC6.3 cells because this method allows the effect of the expression vectors on several reporter constructs to be rapidly screened at one time, something which microinjection does not allow due to the limitations of this technique. pGL3-P, containing the SV40 promoter, was tested as a negative control because this promoter is not activated by the MLK/JNK pathway and consequently its activity was not altered by either of the MLK3 expression vectors. In contrast to this, the *c-jun* promoter, a known target of the MLK/JNK pathway, was activated by the wild type but not the kinase dead MLK3 indicating that the expression vectors were functioning as predicted. The specificity of this activation was confirmed by the observation that there was no difference in the response of a reporter containing the mutated *c-jun* promoter, which is

not activated by the MLK/JNK pathway to either MLK3 constructs. In these pilot experiments, the empty pcDNA3 vector was also transfected to determine the basal level of promoter activity in the absence of any MLK3 expression vector. Comparison of the kinase dead form could be made to this to establish the level of activity with this expression vector. Both MLK3 constructs did cause a small amount of activation of the j1j2 mutant promoter, compared to the empty vector control, but these levels were much lower than the activation of the wild type c-Jun promoter. This suggests that the activation of the j1j2 promoter was a non-specific activation that was not occurring through the jun1 and jun2 sites in the promoter and was MLK3 independent since there was no difference between level with the wild type and kinase dead construct. In microinjection experiments the kinase dead MLK3 was used as the negative control that the induction with active wild type MLK3 was calculated relative to because the only difference between these two constructs was the mutation affecting their activity. This meant that any difference in the response of the reporter construct with these expression vectors was due to alterations in the MLK3 activity rather than any non-specfic effects due to other differences in co-injected vectors.

The activity of the MLK/JNK pathway in sympathetic neurons after NGF deprivation can be inhibited by treating the cells with the small molecule MLK inhibitors CEP-1347 and CEP-11004 (Wang *et al.*, 2005). Use of these inhibitors results in long term neuronal survival and metabolism after NGF deprivation (Harris *et al.*, 2002) reflecting the importance of the MLK/JNK pathway in neuronal apoptosis. Like CEP-1347, CEP-11004 at 400 nM dramatically reduces c-Jun protein levels in sympathetic neurons after NGF deprivation (Figure 4.1; Wang *et al.*, 2005), indicating that this is a suitable concentration for inhibiting the MLK/JNK pathway. CEP-1347 has previously been shown to reduce *dp5* induction by approximately 75% after NGF deprivation (Harris and Johnson, Jr., 2001) and my results indicate that CEP-11004 has a similar effect on *dp5* mRNA levels, although the reduction with this compound was less. This could be due to CEP-11004 being a less potent MLK inhibitor than CEP-1347 (Wang *et al.*, 2005), which is reflected by the incomplete inhibition of phospho-c-Jun in Figure 4.1, and leads to higher *dp5* mRNA levels after NGF deprivation when treated with CEP-11004 compared to CEP-1347. Although CEP-1347 may be a more effective

MLK/JNK inhibitor, this compound was no longer available to the research community because of its use in a clinical trial for the treatment of Parkinson's disease (Saporito *et al.*, 2002;Wang *et al.*, 2004). Despite the lower level of potency, CEP-11004 still appears to be an effective JNK inhibitor making it a suitable compound for use in these experiments, although in future work higher levels of this compound could be used, for example 800 nM.

In contrast to the MLK/JNK pathway, the PI3-K pathway is active in sympathetic neurons in the presence of NGF and PI3-K activity falls after NGF withdrawal (Chang *et al.*, 2002). When sympathetic neurons are treated with the specific PI3-K inhibitor LY294002, the cells undergo apoptosis in the presence of NGF indicating the important role of this pathway in neuronal survival (Bartlett *et al.*, 1997). It was important to try and establish whether inhibiting the PI3-K pathway alters the expression of endogenous *dp5* in sympathetic neurons and although this was examined by RT-PCR, the results from these experiments were inconclusive. When the mean level of *dp5* mRNA after treatment with LY294002 is considered it appears that inhibiting PI3-K has little effect on regulating *dp5* expression, suggesting that the increase in *dp5* mRNA level that occurs after NGF deprivation is not due to a reduction in PI3-K activity. If individual experiments are examined the results appear to cluster into two groups: one group shows increased levels of expression after LY294002 treatment whereas the other indicates that *dp5* levels decrease. This difference in trends suggests that the mean result may not reflect what is actually occurring and further work must be carried out to investigate the effect of LY294002 on endogenous *dp5* levels. It is possible that the results form two groups due to experimental differences in the way that samples were treated, although great care was taken to reduce any sources of variation. For example, cells were treated with the same amount of LY294002 for the same period of time. Differences may have occurred after using a new batch of inhibitor or DMSO for dilution. There could have been some variation between different preparations of neurons, although it is unlikely that this should cause such extreme differences in the results. To overcome this problem, a higher concentration of LY294002 could be used to ensure high levels of PI3-K inhibition during the course of the experiment and mRNA levels could be quantified using real time PCR.

The pathways described above alter in response to NGF deprivation and in order to determine whether they are involved in *dp5* regulation after NGF withdrawal, *dp5* reporter constructs known to be regulated by NGF were tested. The constructs used contained the 1 kb of *dp5* promoter sequence from directly upstream of the transcriptional start site, either alone or with sequences from the intron, including a region conserved between the rat, mouse and human sequences, or the 3'UTR. All three of these regions resulted in higher levels of luciferase activity after NGF deprivation suggesting that each contain an element that responds to this stimulus to increase *dp5* expression. Testing the expression of these constructs either after activating MLK3 or inhibiting PI3-K in the presence of NGF or by inhibiting the MLK/JNK pathway following NGF withdrawal would indicate whether increases in *dp5* expression were due to these regions containing elements that respond to changes in the activity of these signalling pathways after NGF deprivation.

Activating the MLK3 pathway by co-injecting the wild type MLK3 expression vector with 1kb*dp5*-LUC, suggested that the *dp5* promoter region contained an element that was regulated by the MLK/JNK pathway although individual results did show some variability indicating that further examination of the role of this pathway was required. Therefore, CEP-11004 was used to inhibit MLK activity after NGF deprivation to determine whether this altered the response of 1kb*dp5*-LUC. This experiment demonstrated that in the presence of CEP-11004 the induction of the construct containing the promoter was reduced, supporting the hypothesis that this region contains sequences that respond to the MLK/JNK pathway. The PI3-K pathway does not appear to regulate elements within the 1kb promoter sequence since inhibition of this pathway by treatment with LY294002 did not increase the activity of 1kb*dp5*-LUC.

The construct 1kb*dp5*-LUC+I(F) contains a relatively short sequence from the *dp5* intron in addition to the 1 kb of promoter sequence. Because the promoter region appears to be upregulated by an increase in MLK/JNK pathway activity, as would occur after NGF deprivation, it would be predicted that this construct would also be regulated in this way. This construct does appears to behave in a similar manner to 1kb*dp5*-LUC with respect to the JNK pathway although it is not possible to establish whether the intron sequence itself contains any JNK-regulated regions because any response from

this region alone is masked by the response due to the promoter. There does not appear to be a noticeable additional response with the presence of the intron sequence but this region could be tested alone in future experiments to determine whether it contains any MLK/JNK signalling targets. This could be done by testing the intron sequence in the presence of an alternative promoter, although this construct must respond in the same way to NGF deprivation to ensure that the intron region was behaving as it did in the context of the *dp5* promoter. This did not appear to be the case when this region was inserted upstream of the SV40 promoter (see Chapter 3) as this construct was not regulated by NGF withdrawal, indicating the importance of using a suitable construct. Alternatively, if the JNK target sequence within the *dp5* promoter was identified, this could be inactivated by mutation in 1kb*dp5*-LUC+I(F) allowing the effect of the intron alone to be determined whilst maintaining the intron in a functional context. 1kb*dp5*-LUC+I(F) also behaved in a similar manner to 1kb*dp5*-LUC with respect to PI3-K inhibition since this construct was not affected by LY294002, suggesting that the intron region does not contain a sequence that is regulated by the PI3-K pathway.

Again, when testing 1kb*dp5*-LUC+3'UTR it would be expected that this would behave in a similar manner to 1kb*dp5*-LUC since this construct also contained the 1 kb promoter region. This was not the case following overexpression of wild type MLK3. Unlike 1kb*dp5*-LUC, the construct containing the additional sequence from the 3'UTR was not activated by overexpressed MLK3 in the presence of NGF. However inhibiting the MLK/JNK pathway by treatment with CEP-11004 did reduce the activity of 1kb*dp5*-LUC+3'UTR after NGF deprivation, as would be expected if this construct contained a region that was regulated by the JNK pathway. These conflicting results could be due to the non-specific effects of the inhibitor CEP-11004. Both CEP-1347 and CEP-11004 have been found to activate the PI3-K pathway, increasing the activation of the downstream target Akt (Roux *et al.*, 2002;Wang *et al.*, 2005). Activation of this pathway occurs as an indirect result of these inhibitors increasing the expression of the NGF receptor TrkA and causing ligand-independent receptor activation. This results in the activation of the downstream PI3-K pathway which contributes to the increased survival that occurs after treatment with CEP-11004 following NGF withdrawal (Wang *et al.*, 2005). It is therefore possible that the conflicting results obtained for the

construct 1kb*dp5*-LUC+3'UTR could be due to an effect of CEP-11004 on the PI3-K pathway, a hypothesis which is supported by the result that this construct is activated by PI3-K inhibition, suggesting that a sequence in the 3'UTR is regulated by the PI3-K pathway.

A possible model of *dp5* regulation by the MLK/JNK and PI3-K signalling pathways is illustrated in Figure 4.9. This model proposes that in the presence of NGF *dp5* levels remain low because the PI3-K pathway targets a sequence within the 3'UTR. This sequence could, in the presence of active PI3-K, either bind a repressor that inhibits *dp5* promoter activity, or the 3'UTR of *dp5* mRNA might bind a protein that decreases RNA stability. After NGF withdrawal, which leads to a decrease in PI3-K activity, the repressive effect of the 3'UTR region is relieved allowing promoter activity or RNA stability to increase, which results in higher *dp5* levels. In parallel to this, activation of the MLK/JNK pathway after NGF withdrawal leads to further increases in *dp5* expression through elements within the promoter and possibly the intron. In this model, inhibiting PI3-K would not alter the activity of the constructs 1kb*dp5*-LUC or 1kb*dp5*-LUC+I(F) because this pathway does not directly affect any sequences within these regions, whereas the activity of 1kb*dp5*-LUC+3'UTR would increase after PI3-K inhibition due to the repressive effect of this pathway on the 3'UTR element being relieved. Activation of the MLK/JNK pathway would increase the activity of 1kb*dp5*-LUC and 1kb*dp5*-LUC+I(F) because both of these constructs contain the promoter element that was activated by this pathway. Although 1kb*dp5*-LUC+3'UTR also contains this region, the presence of the 3'UTR prevents promoter activation, even after MLK3 overexpression because this sequence contains an element that represses promoter activity or reduces RNA stability in the presence of active PI3-K. All three of these *dp5* reporter constructs would show decreased induction after NGF withdrawal following treatment with CEP-11004; inhibition of the MLK/JNK pathway would prevent 1kb*dp5*-LUC and 1kb*dp5*-LUC+I(F) increasing in activity whereas activation of the PI3-K pathway by this compound could account for the decrease in activity of 1kb*dp5*-LUC+3'UTR after NGF deprivation. In future experiments the effect of the 3'UTR on the promoter in the presence of the intron sequence needs to be determined. This should be tested by repeating the microinjection experiments in this chapter with

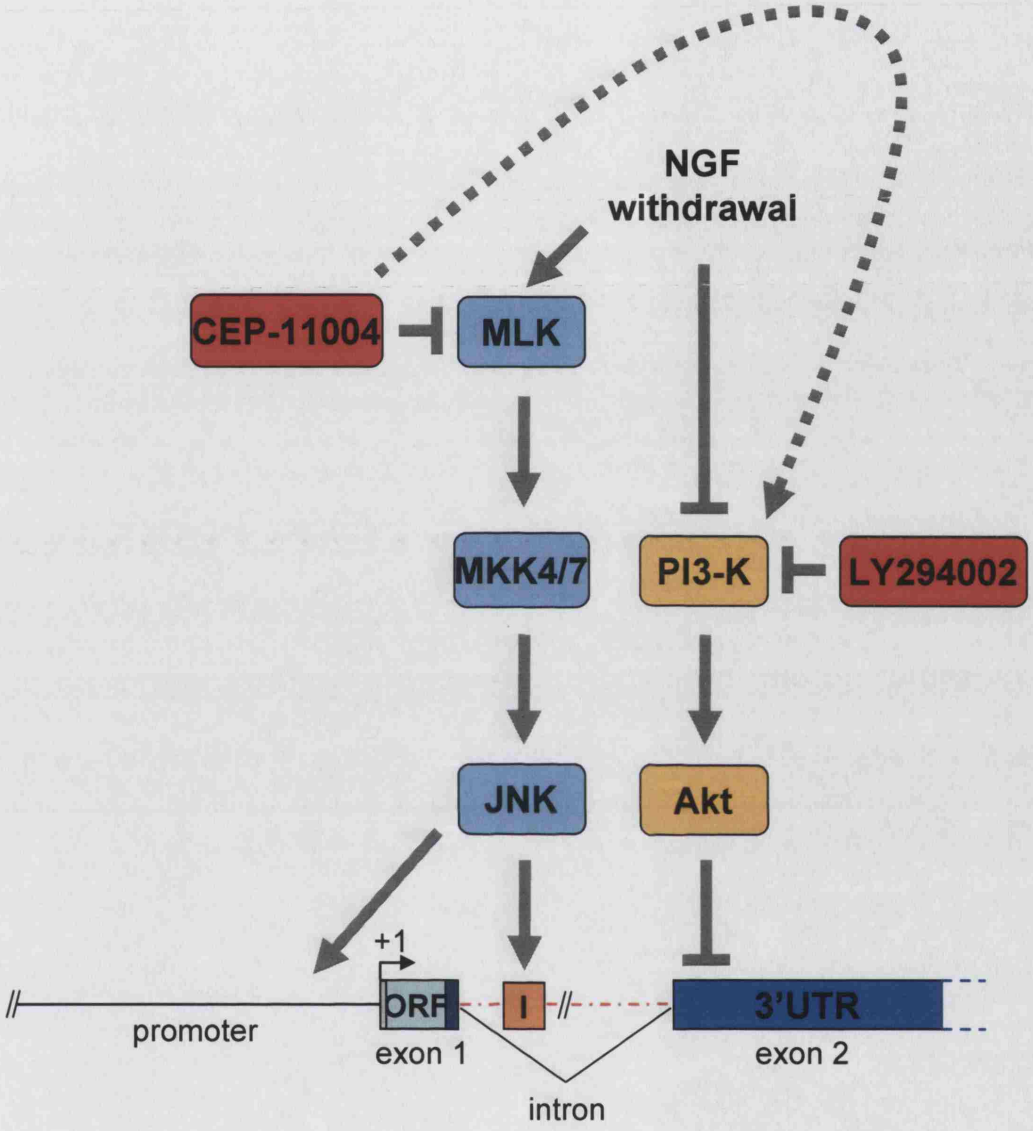


Figure 4.9 A potential mechanism for the regulation of *dp5* in sympathetic neurons following NGF deprivation

After NGF withdrawal, activation of the MLK/JNK pathway upregulates *dp5* expression mediated by regulatory elements located in the promoter and the conserved region of the intron. Parallel to this, NGF deprivation leads to a decrease in PI3-K activity which leads to *dp5* upregulation due to its inhibitory effect on the regulatory region in the 3'UTR. CEP-11004 has been reported to increase TrkA expression in sympathetic neurons, leading to NGF-independent activation of TrkA and PI3-kinase (Wang *et al.*, 2005).

the construct 1kb*dp5*-LUC+ALL. This construct contains all three regions and the response of this construct to the MLK/JNK and PI3-K pathways would more accurately represent the response of the endogenous *dp5* gene.

There were some limitations to the experiments performed in this chapter, largely due to restrictions in the number of constructs that could be microinjected in each individual experiment. It would be beneficial to test all reporter constructs that were being compared in one experiment but this was not possible due to the large number of injections this required for each of the test conditions with each construct. It was therefore important to ensure that an appropriate number of individual experiments were performed so that the result obtained was more likely to be a true representative of what was actually occurring. This could also be overcome in part by using multiple methods to investigate the role of each pathway, for example both activation and inhibition of a particular pathway. For each construct in each test condition 150 neurons were microinjected and it is possible that this number could be reduced to allow the injection of a greater number of constructs within the same experiment, as it has been demonstrated that the response of one microinjected neuron can be detected by dual luciferase assay (Pajak *et al.*, 2003).

The results in this chapter provide some evidence for the involvement of different *dp5* regions in the regulation *dp5* expression by the JNK and PI3-K pathways. Future work should involve the further identification of the components involved in these pathways that lead to *dp5* regulation as well as localisation of the exact regions of the *dp5* gene involved. The problems encountered with the non-specificity of CEP-11004 could be overcome by use of the direct JNK inhibitor SP600125, which can effectively prevent NGF deprivation-induced apoptosis in sympathetic neurons (Besirli and Johnson, Jr., 2003). In addition to testing the results obtained using the MLK3 expression vectors, it would determine whether the effects of activating or inhibiting MLK were due to alterations in downstream JNK activity. The JNK-binding domain of JNK interacting protein 1 (JIP-1) is a potent JNK inhibitor that is able to inhibit c-Jun phosphorylation, *c-jun* promoter activation and NGF deprivation-induced apoptosis (Eilers *et al.*, 2001). This could also be used in microinjection experiments to determine whether MLK signalling was occurring via JNK activation. The role of other

downstream components of the MLK signalling pathway could also be studied. For example, the involvement of the transcription factor c-Jun could be established through use of the dominant negative c-Jun expression vector that is known to protect sympathetic neurons against NGF withdrawal-induced death (Ham *et al.*, 1995). In a similar manner, downstream targets of the PI3-K pathway, such as Akt and the Forkhead transcription factors could be tested using appropriate expression vectors. It would also be useful to investigate the role of other signalling pathways such as the ERK pathway to fully understand how *dp5* is regulated following NGF deprivation.

5 Characterisation of the *dp5* proximal promoter region and identification of an E4BP4-binding site

5.1 Introduction

The rate of transcription of eukaryotic genes is not only regulated by the binding of gene-specific transcription factors that associate with enhancer sequences located many kilobases upstream or downstream of the transcriptional start site, but the core promoter sequence itself, which binds the multiprotein complex TFIID to initiate transcription, also plays a critical role. The core promoter is a relatively short sequence of ~60 bp surrounding the transcriptional start site that classically contains a TATA box located at between -25 and -30 bp and may contain an initiator element (INR) and downstream promoter element (DPE) (Levine and Tjian, 2003;Smale, 1997). In addition, many genes contain binding sites for regulatory elements immediately 5' of the core promoter, which alter the transcriptional activity of the promoter.

The transcription factor E4BP4 (also called NFIL3) is an example of a regulatory factor that appears to repress transcriptional activity by interfering with the general transcription machinery, possibly via the TATA-binding protein (TBP)-binding repressor protein Dr_1 (Cowell and Hurst, 1996). E4BP4 is a widely expressed mammalian basic leucine zipper (bZIP) transcription factor which has overlapping DNA binding specificity with CREB and ATF family members (Cowell *et al.*, 1992). This protein has been implicated in a wide range of processes, including cell survival in pro-B lymphocytes and regulation of the mammalian circadian oscillatory mechanism, and has been shown to function as both a transcriptional repressor and activator, depending on context (Ikushima *et al.*, 1997;Mitsui *et al.*, 2001;Zhang *et al.*, 1995).

In *C. elegans*, the bZIP transcription factor CES-2 plays an important role during the development of neurosecretory motoneurons (NSM). NSM precursor cells divide to form two daughter cells but only one survives to become an NSM as the other sister cell undergoes programmed cell death. In mutants lacking *ces-2* both sister cells survive indicating that this bZIP protein is required for developmental apoptosis in this cell type

in the nematode (Ellis *et al.*, 1991a). Like CES-2 in *C. elegans*, E4BP4 has been implicated in the regulation of motoneuron survival during vertebrate development, although in contrast to CES-2 it appears to function as an antiapoptotic factor. E4BP4 expression is highest in motoneurons that survive periods of developmental cell death and overexpression of E4BP4 protects motoneurons cultured *in vitro* against apoptosis induced by neurotrophic factor deprivation and death receptor activation, and overexpression *in vivo* it reduces the number of motoneurons that die during development (Junghans *et al.*, 2004).

Identification of a minimal promoter that is sufficient for full promoter activity, as well as the important elements within this region, can be useful in determining the mechanism by which transcription is regulated. Previously, a series of *dp5* reporter constructs had been made and tested to determine the effect of NGF withdrawal on their activity (see Chapter 3). The basal activity of these constructs and constructs containing sequential 5' deletions of the *dp5* promoter region, was examined in this chapter. Inspection of the DNA sequence upstream of the *dp5* transcriptional start site identified several potential transcription factor binding sites, including an E4BP4-binding site and the results of experiments investigating the possible role of this site are also presented.

In this chapter some repeated microinjection experiments were performed by my supervisor Dr. Jonathan Ham.

5.2 Results

5.2.1 The basal activity of some *dp5* reporter constructs differs in PC6.3 cells and sympathetic neurons

To determine the contribution of different regions of the *dp5* gene to basal promoter activity, constructs containing these regions were transiently transfected into neuronally differentiated PC6.3 cells. The transfected constructs are described in Chapter 3 and were as follows: 1kb*dp5*-LUC, 150bp*dp5*-LUC, 1kb*dp5*-LUC+I(F), 1kb*dp5*-LUC+3'UTR, 1kb*dp5*-LUC+A(F), 1kb*dp5*-LUC+A1, 1kb*dp5*-LUC+A2, 1kb*dp5*-LUC+A(R) and 1kb*dp5*-LUC+ALL. The *Renilla* luciferase construct pRL-TK was also co-transfected with each firefly luciferase construct. Luciferase activity was determined

24 hours after transfection by dual luciferase assay and firefly luciferase activity was normalised to *Renilla* luciferase activity (firefly luciferase activity ÷ *Renilla* luciferase activity). The activity of each construct was calculated relative to that of 1kb*dp5*-LUC, which was set as 1. The basal activity of each construct in PC6.3 cells is illustrated in Figure 5.1A. Reporter constructs containing either 1 kb or 150 bp of the *dp5* promoter sequence had a similar level of activity, suggesting that the *dp5* minimal promoter is contained within 150 bp. The presence of the conserved intron sequence did not have a large effect on the activity of the 1 kb promoter but, in contrast to this, addition of the *dp5* 3'UTR caused a ~89% decrease in basal promoter activity. Part of this decrease may have been due to sequences located in the first 645 bp of the 3'UTR, possibly within the sequence from exon 1, since the presence of fragments A and A1 caused a 31% and 21% drop in activity respectively. This decrease in promoter activity recorded in the presence of fragment A may depend on the orientation of this fragment because the promoter activity decreased more when this sequence was in the reverse orientation. For the further drop in activity seen with 1kb*dp5*-LUC+3'UTR, the remainder of the 3'UTR sequence that is not present in fragment A appears to be required. The construct containing all three regions of the *dp5* gene (1kb*dp5*-LUC+ALL) had a similar basal activity to the construct containing the promoter and 3'UTR, suggesting that sequences from within the 3'UTR have a repressive effect on promoter activity, and that this effect was not altered by the presence of the intron sequence.

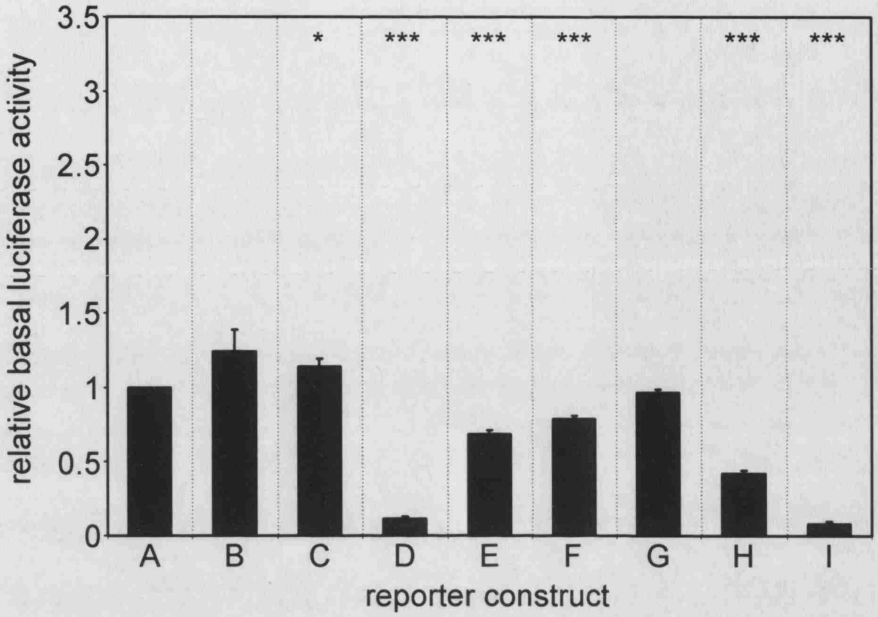
The same set of reporter constructs had been tested previously in microinjection experiments to compare the response of each to NGF withdrawal (see Chapter 3). To determine the basal activity of each construct in sympathetic neurons, the results obtained previously were used and the normalised activity of these constructs in the presence of NGF was compared. In each of the experiments used for this analysis, 1kb*dp5*-LUC had been used as a control, therefore the activity of each construct was calculated relative to this control and the compiled results from individual experiments were compared (Figure 5.1B). As in PC6.3 cells, these results suggest that the *dp5* minimal promoter is contained within 150 bp since the activity of the construct containing the shorter promoter sequence (150bp*dp5*-LUC) does not decrease compared to the construct with the longer promoter sequence. In sympathetic neurons the 150 bp

Figure 5.1 Comparison of the basal activity of *dp5* reporter constructs in PC6.3 cells and sympathetic neurons

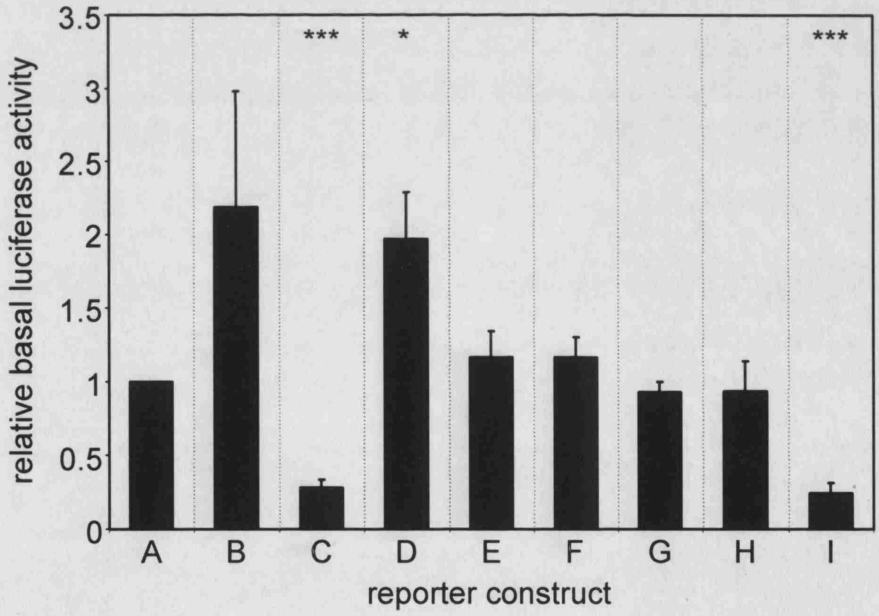
A) Neuronally differentiated PC6.3 cells were transiently transfected with equimolar amounts of different *dp5* luciferase reporter constructs (1kb*dp5*-LUC was transfected at 0.17 μg/μl) in addition to the *Renilla* luciferase construct pRL-TK [0.05 μg/μl]. Luciferase activity was determined 24 hours after transfection using a dual luciferase assay. Firefly luciferase activity was normalised for *Renilla* luciferase activity and the activity of each construct was calculated relative to 1kb*dp5*-LUC, which was set as 1. Results were combined from two different experiments. Each of these was performed at least three times in duplicate and the results ± the standard error of the mean (SEM) are shown (* $P<0.05$, *** $P<0.001$, *t*-test).

B) The basal activity of the *dp5* luciferase reporter constructs microinjected in Chapter 3 was determined. Each construct was microinjected as outlined in Chapter 3. The firefly luciferase activity of each construct in the presence of NGF was calculated relative to the to the activity of 1kb*dp5*-LUC in individual experiments, which was set as 1. The compiled results from different experiments ± the SEM are shown. Each experiment was repeated at least 3 times (* $P<0.05$, *** $P<0.001$, *t*-test).

A transient transfection



B microinjection



A = 1kb*dp5*-LUC	F = 1kb*dp5*-LUC+A1
B = 150bp*dp5*-LUC	G = 1kb*dp5*-LUC+A2
C = 1kb*dp5*-LUC+I(F)	H = 1kb*dp5*-LUC+A(R)
D = 1kb*dp5*-LUC+3'UTR	I = 1kb*dp5*-LUC+ALL
E = 1kb*dp5*-LUC+A(F)	

promoter sequence appears to have a higher activity on average although there does seem to be a degree of variation with this result as reflected by the error bars. Unlike the result obtained in PC6.3 cells, the addition of the intron to the 1 kb promoter represses promoter activity, causing a reduction of ~76%. Also, in contrast to PC6.3 cells, the presence of the 3'UTR does not decrease promoter activity since 1kb*dp5*-LUC+3'UTR has 1.97 fold higher levels of activity compared to 1kb*dp5*-LUC. The remaining *dp5* reporter constructs appear to behave in a similar manner to 1kb*dp5*-LUC, apart from 1kb*dp5*-LUC+ALL, which again has the lowest level of basal activity (~93% decrease). These results indicate that *dp5* reporter constructs do not always respond in the same way in transfected PC6.3 cells and microinjected sympathetic neurons emphasising the importance of confirming results in primary cells. The construct 1kb*dp5*-LUC+ALL did have the same response compared to 1kb*dp5*-LUC in both systems suggesting that PC6.3 cells may be a reasonable model for testing the activity of 1kb*dp5*-LUC+ALL in different conditions.

5.2.2 Identification of potential transcription factor binding sites

It is likely that expression of the *dp5* gene is regulated in part by transcription factors that bind to sequences within the *dp5* promoter and alter rates of transcription. Since many transcription factors bind to specific DNA sequences, an initial step towards identifying potentially important binding proteins involves examining the DNA sequence for any possible protein binding sites. Although sequences can be examined by eye to identify potentially interesting sites, this is an inefficient method since large sequences cannot be easily analysed, elements controlling transcriptional regulation are often located away from transcription initiation sites and many transcription factors are able to bind to sequences that vary considerably from the consensus sequence. These problems have been largely overcome through the application of bioinformatics, involving the analysis of DNA sequences by specifically designed computer programmes that can search large regions of DNA for homology to known transcription factor binding sites with a programmed degree of variation from the consensus. Such systems depend on a reliable database of protein binding sites for accurate analysis.

An alternative approach for identifying larger regions involved in gene regulation is the comparison of orthologous gene sequences. This process, known as phylogenetic footprinting, relies on the principle of the preferential conservation of functional sequences throughout evolution. Since mutations that appear in functionally important sites are more likely to be disruptive, there is a difference in the rate of evolution between functional and non-functional regions of the genome, with functional regions being more highly conserved between species. These regions are more likely to mediate biological function and their identification can be combined with computational sequence analysis of consensus sites to improve the prediction of functionally important transcription factor binding sites. This approach is used by the ConSite programme (available at http://www.phylofoot.org/ and described in Lenhard *et al.*, 2003) which analyses DNA sequences using a specific algorithm for detecting transcription factor binding sites based on phylogenetic footprinting. This programme uses a database of metazoan transcription factor binding profiles compiled from *in vivo* and *in vitro* data from the biological research literature, with on average 31.2 sites contributing to the binding profile of each transcription factor. The ConSite programme also allows a degree of user input to set parameters of conservation and consensus homology. Therefore, this programme was used to analyse regions of the *dp5* gene, including the promoter sequence upstream of the transcriptional start site, using a comparison of the rat *dp5* and human *hrk* promoter sequences. An example of the output obtained from this analysis is illustrated in Figure 5.2, which identifies a number of potential sites within the region 109 to 50 bases upstream of the transcriptional start site in the *dp5* promoter. A number of potentially interesting transcription factor binding sites were identified and are discussed in Section 5.2.3.

5.2.3 Deletion of potential transcription factor binding sites reduces basal promoter activity

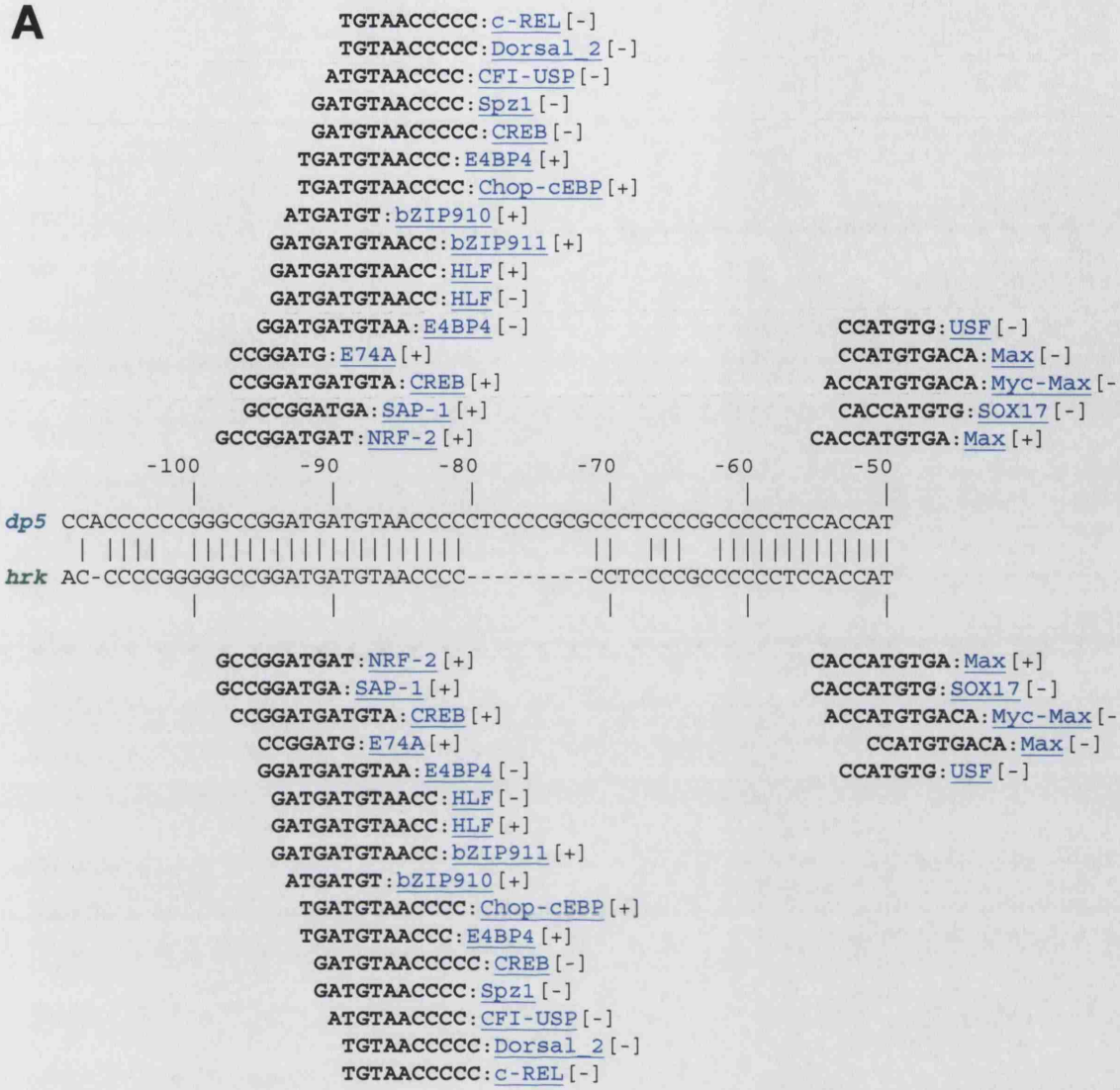
Reporter constructs containing different lengths of the *dp5* promoter sequence inserted upstream of the luciferase gene in pGL3-basic were made by Dr. Jonathan Gilley. Sequences of approximately 4.5 kb, 1.6 kb, 1 kb, 350 bp, 200 bp and 150 bp from directly upstream of the transcriptional start site were chosen because these lengths

Figure 5.2 The ConSite algorithm was used to analyse the *dp5* and *hrk* sequences for the presence of potential transcription factor binding sites

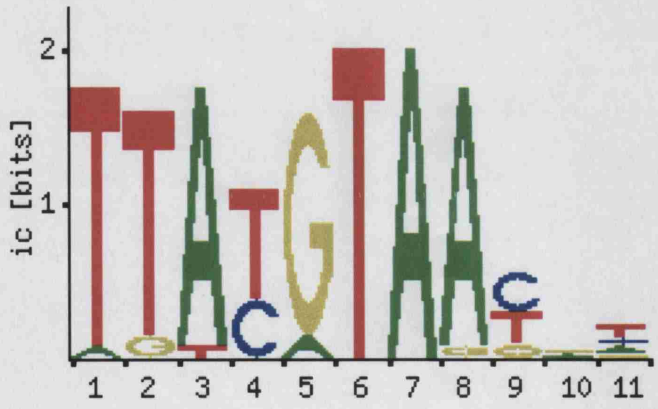
A) An example of the output from an analysis performed with the *dp5* and *hrk* promoters. Sequence conservation cutoff was set at 80% and transcription factor score cutoff was set at 75% with a window size of 10. Distance from the transcriptional start site is indicated.

B) An example of the binding profile of the human transcription factor E4BP4, compiled from 23 sites in the biological literature. The base frequency at each position is also shown.

A



B



A	[1	0	22	0	2	0	23	22	0	7	5]
C	[0	0	0	8	0	0	0	0	11	5	5]
G	[0	2	0	0	21	0	0	1	4	7	3]
T	[22	21	1	15	0	23	0	0	8	4	10]

corresponded to restriction enzyme sites within the promoter region (4.5kb*dp5*-LUC, 1.6kb*dp5*-LUC, 1kb*dp5*-LUC, 350bp*dp5*-LUC, 200bp*dp5*-LUC and 150bp*dp5*-LUC respectively; Figure 5.3A). After closer examination of the promoter region from -102 to +40, used in 150bp*dp5*-LUC, sequences were found that closely matched the consensus sequences of some transcription factor binding sites. These included two ATF-like sites, one of which was an exact match to the consensus binding site for the transcription factor E4BP4, a GC box, an AP-1-like site, and a region that was AT-rich (Figure 5.3B). To determine whether any of these sites contributed to basal promoter activity, reporter constructs were made in which the sites were sequentially deleted from the *dp5* promoter upstream of the luciferase gene. The constructs deleting the E4BP4-like site, GC box, AP-1-like site and AT-rich region were named Δ-66*dp5*-LUC, Δ-51*dp5*-LUC, Δ-43*dp5*-LUC and Δ-25*dp5*-LUC respectively (Figure 5.3C), corresponding to the point of deletion upstream of +1.

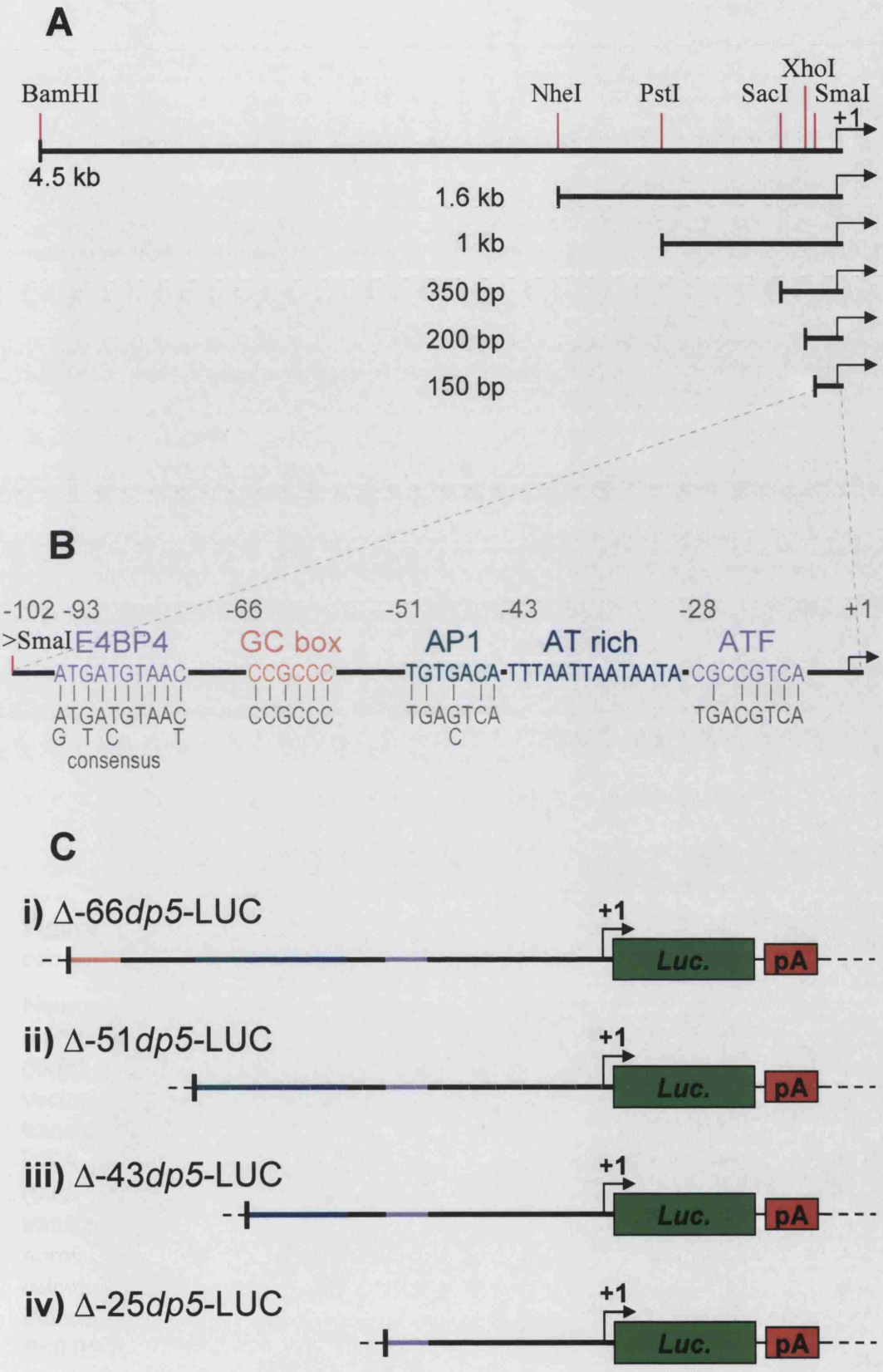
To determine the basal activity of the different *dp5* promoter deletion constructs, neuronally differentiated PC6.3 cells were transiently transfected with equimolar amounts of each *dp5* reporter construct or the promoterless construct pGL3-basic, as well as the *Renilla* luciferase construct pRL-TK. Luciferase activity was determined 24 hours after transfection using a dual luciferase assay and normalised firefly luciferase activity was calculated as described above. The activity of each *dp5* reporter construct was calculated relative to empty pGL3-basic and the results are shown in Figure 5.4. Varying the length of promoter sequence from 4.5 kb to 150 bp did not appear to have a large effect on activity, apart from a decrease (42%) and slight increase (139%) in the activity of the constructs 4.5kbdp5-LUC and 200bpdp5-LUC respectively, when compared to 1kb*dp5*-LUC. As seen previously, the construct containing 150 bp of the promoter had a similar activity to the construct containing the 1 kb promoter sequence but deletion of 36 base pairs from the 5' end of the 150 bp sequence, as in the construct Δ-66*dp5*-LUC, resulted in a significant 68% decrease in basal activity ($p<0.001$). Deletion of an additional 15 bases, including the GC box, further decreased the basal activity to the level of the empty vector pGL3-basic, suggesting that both of these regions contain sequences that contribute to the basal activity of the *dp5* promoter. The effect of deleting the other potential sites of interest could not be determined because

Figure 5.3 Structure of *dp5* promoter deletion mutants

A) Luciferase reporter constructs were made by Dr. Jonathan Gilley containing different lengths of *dp5* sequence from upstream of the transcriptional start site (4.5 kb, 1.6 kb, 1 kb, 350 bp, 250 bp and 150 bp). These lengths were chosen as they corresponded to restriction enzyme sites and each sequence was inserted upstream of the firefly luciferase gene in pGL3-basic.

B) Potential transcription factor binding sites were identified in the 150 bp region of promoter upstream of the *dp5* initiator codon. These included two potential ATF sites, one of which matched the consensus E4BP4 binding site, as well as a GC-box, a potential AP-1 binding site and an AT-rich region.

C) Luciferase reporter constructs were made containing sequential deletions of the potential E4BP4 binding site, GC-box, AP-1 site and AT rich region upstream of the firefly luciferase gene in pGL3-basic. Each construct also contained the SV40 poly-A termination signal.


A
BamHI
NheI
PstI
SacI
XhoI
SmaI
+1
4.5 kb
1.6 kb
1 kb
350 bp
200 bp
150 bp
B
-102 -93
-66
-51
-43
-28
+1
>SmaI E4BP4
GC box
AP1
AT rich
ATF
ATGATGTAAC
CCGCCC
TGTGACA-TTTAATTAATAATA-CGCCGTCA
ATGATGTAAC
G T C T
consensus
CCGCCC
TGAGTCA
C
TGACGTCA
C
i) Δ-66dp5-LUC
ii) Δ-51dp5-LUC
iii) Δ-43dp5-LUC
iv) Δ-25dp5-LUC
+1
Luc.
pA

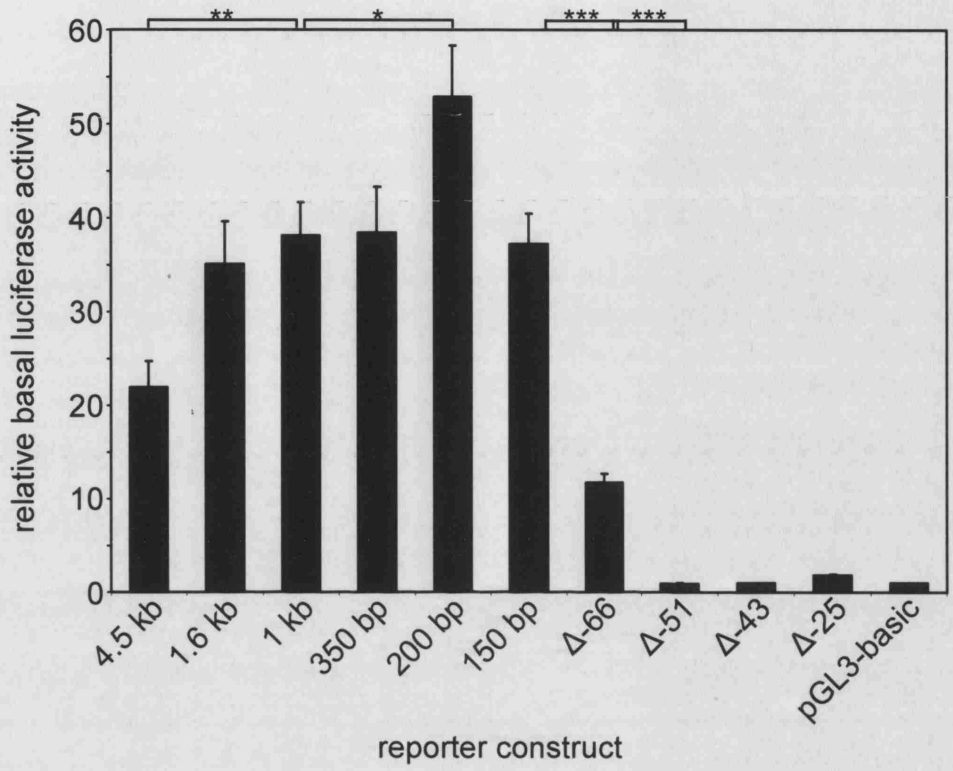


Figure 5.4 Comparison of the basal activity of luciferase reporter constructs containing different lengths of the *dp5* promoter

Neuronally differentiated PC6.3 cells were transiently transfected with luciferase reporter constructs containing different length of the *dp5* promoter (including Δ-66*dp5*-LUC, Δ-51*dp5*-LUC, Δ-43*dp5*-LUC and Δ-25*dp5*-LUC). The empty vector pGL3-basic was also transfected as a control. The constructs were transfected at equimolar amounts, with 4.5kb*dp5*-LUC transfected at 0.2 μg of DNA per well. The *Renilla* luciferase construct pRL-TK was also transfected (0.05 μg per well). Luciferase activity was determined 24 hours after transfection using a dual luciferase assay. Firefly luciferase activity was normalised for *Renilla* luciferase activity and the activity of each construct was calculated relative to pGL3-basic, which was set as 1. The results of 3 independent experiments, each performed in duplicate ± the SEM are shown (* $P<0.05$, ** $P<0.01$, *** $P<0.001$, *t*-test).

these constructs lacked the GC box making any additional decrease due to the deletion of other sequences impossible to detect.

5.2.4 *e4bp4* mRNA is expressed in sympathetic neurons but is not regulated by NGF deprivation

The region between -102 and -66 bp upstream of the *dp5* transcription initiation site contains a sequence which matches the binding site for the transcription factor E4BP4 and deletion of the region containing this site appears to cause a decrease in basal promoter activity. Before examining whether this transcription factor could play a role in regulating *dp5* transcription it was important to establish whether E4BP4 was expressed in sympathetic neurons. This was performed by examining mRNA expression by RT-PCR analysis. Sympathetic neurons were cultured *in vitro* for 5 to 7 days and then maintained in the presence or absence of NGF. RNA was extracted at different time points after NGF withdrawal (0, 4, 8, 16 and 24 hours) and RT-PCR analysis was performed using specific primers to determine whether *e4bp4* was expressed and if so, whether the level of expression altered after NGF deprivation. Following quantification, the level of *e4bp4* expression at each time point both + and - NGF was calculated relative to the level at 0 hours and values were adjusted for neurofilament expression to control for variations in the total amount of mRNA. The results show that *e4bp4* mRNA is expressed in sympathetic neurons both in the presence and absence of NGF (Figure 5.5A) and that, although the level of expression does vary slightly after changing the medium, there is no difference in the pattern of expression in cells cultured in the different conditions (Figure 5.5B). Therefore the level of *e4bp4* mRNA does not appear to be regulated by NGF deprivation. However, this does not exclude the possibility that NGF regulates the level of E4BP4 protein or its subcellular localisation by post-transcriptional mechanisms.

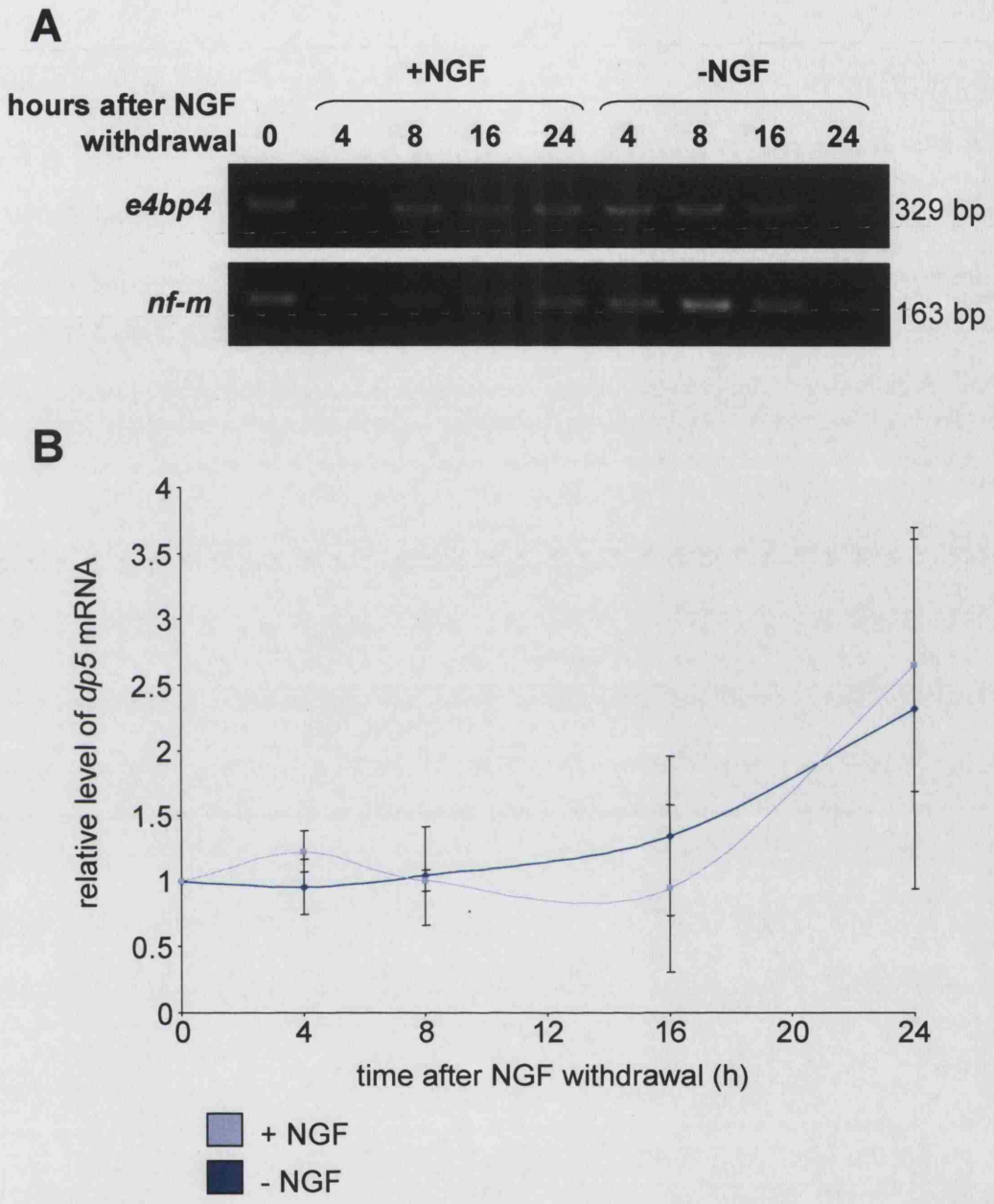


Figure 5.5 RT-PCR analysis of *e4bp4* mRNA levels in sympathetic neurons cultured in the presence and absence of NGF

Sympathetic neurons were maintained in the presence or absence of NGF for different periods of time. RNA was extracted 0, 4, 8, 16 and 24 hours after NGF withdrawal and RT-PCR analysis carried out using *e4bp4* and neurofilament (*nf-m*) specific primers (**A**). The *e4bp4* RNA level at each time point ± NGF was calculated relative to the level at 0 hours, which was set as 1 (**B**). The mean of 3 experiments is shown ± the SEM.

5.2.5 E4BP4 can bind to the sequence in the *dp5* promoter and binding is abolished by the introduction of point mutations

Although *e4bp4* mRNA was expressed in sympathetic neurons, whether E4BP4 protein is capable of binding to the potential binding site in the *dp5* promoter needed to be established. To determine this, an electrophoretic mobility shift assay (EMSA) was performed. Oligonucleotides were designed including the possible E4BP4-binding site and surrounding bases from the *dp5* promoter. In addition, oligonucleotides incorporating three point mutations which have been shown to abolish E4BP4 binding (Cowell *et al.*, 1992), as well as a polymorphism, which should not affect binding, were designed (Figure 5.6A). The E4BP4 protein used in the EMSA was made by *in vitro* translation. Successful translation of this protein was determined by immunoblotting, which indicated that a protein of the expected size had been translated with the E4BP4 template and no protein was present in the empty vector control (Figure 5.6B).

The EMSA in Figure 5.6C was performed by analysing the binding of increasing amounts of *in vitro* translated E4BP4 to the radiolabelled wild type and mutated oligonucleotides described above. Unbound oligonucleotides ran towards the bottom of the gel but, in the presence of E4BP4, two major protein/DNA complexes were observed. One of these corresponded to E4BP4 bound to DNA since it was not observed in the unprogrammed lysate (pcDNA3.1) and was not present when the mutated oligonucleotide was used. The other band was the result of a non-specific interaction, since it was found in all reactions containing lysate. This result indicates that E4BP4 can bind to the DNA sequence containing the E4BP4-binding site found in the *dp5* promoter and that this binding can be abolished by the introduction of three point mutations in this site.

5.2.6 Overexpression of E4BP4 represses the activity of *dp5* reporter constructs containing the E4BP4-binding site in PC6.3 cells

To determine whether overexpression of E4BP4 could alter the activity of the *dp5* promoter, PC6.3 cells were co-transfected with different *dp5* reporter constructs and

Figure 5.6 Binding of E4BP4 to the potential *dp5* E4BP4-binding site

A) Sequence of the oligonucleotides used for EMSA experiments. The wild type E4BP4 site is shown together with a mutated sequence, in which three point mutations were incorporated that would be predicted to abolish the binding of E4BP4 *in vitro* (Cowell *et al.*, 1992).

B) Immunoblot of *in vitro* translated E4BP4 and an empty vector control translation (pcDNA3.1).

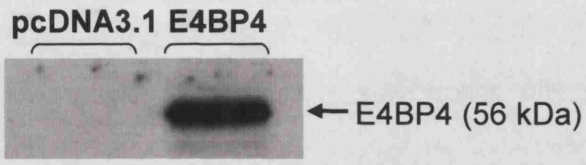
C) Radiolabelled double stranded oligonucleotides corresponding to the *dp5* sequence containing a potential E4BP4-binding site, as well as oligonucleotides containing three point mutations within this site, were incubated with *in vitro* translated E4BP4 or an empty vector control. The binding reactions were analysed by gel electrophoresis and visualised using phosphorimaging.

A

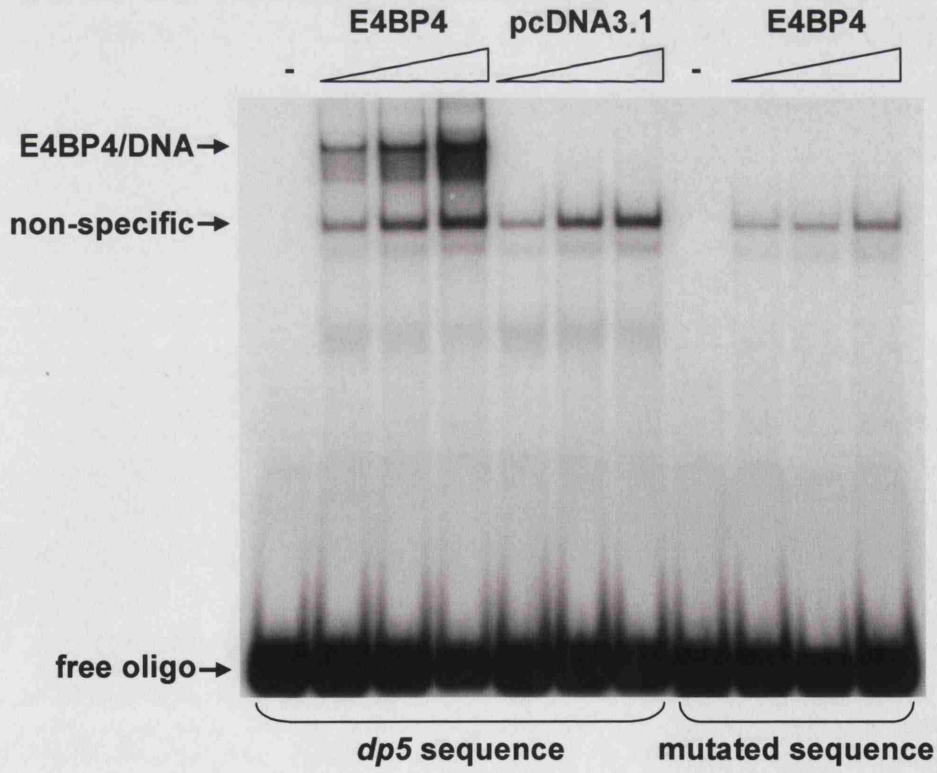
dp5 sequence GCCGGATGATGTAACCCCCT

mutated sequence GCCGGATAACGTCTCCCCCT

B



C



increasing amounts of an E4BP4 expression vector. The *dp5* constructs 1kb*dp5*-LUC+ALL, 1kb*dp5*-LUC, 150bp*dp5*-LUC and Δ-66*dp5*-LUC were tested as well as the vector pGL3-P, which contains the SV40 promoter. The reporter constructs were co-transfected with either 0, 0.1, 0.2, 0.5 or 1 μg of E4BP4 expression vector, each made up to 1 μg with the empty vector pcDNA3.1, and the *Renilla* luciferase construct pRL-TK. Luciferase activity was determined after 24 hours using a dual luciferase assay and firefly luciferase activity was normalised using *Renilla* luciferase activity. The dose response of each construct to increasing amounts of E4BP4 expression vector was calculated and is shown in Figure 5.7. The construct pGL3-P does not contain any known E4BP4-binding sites and the activity of the SV40 promoter is only reduced a small amount by overexpression of E4BP4. The activity of the three *dp5* constructs that contain the E4BP4-binding site (1kb*dp5*-LUC+ALL, 1kb*dp5*-LUC and 150bp*dp5*-LUC) does alter following E4BP4 overexpression. A reduction in activity can be seen after the addition of only 0.1 μg of E4BP4 expression vector (33-47% decrease) and the activity continues to drop with increasing amounts of co-transfected E4BP4 (66-75% decrease after the addition of 1 μg E4BP4). In contrast to this, Δ-66*dp5*-LUC contains a shorter promoter sequence that lacks the E4BP4-binding site and this construct does not respond in the same manner in the presence of E4BP4. The relative luciferase activity recorded in cells transfected with this construct follows a similar pattern to those transfected with pGL3-P, having a maxiumum decrease of 22%. This result suggests that overexpression of E4BP4 can repress *dp5* promoter activity in PC6.3 cells and the 36 base pairs that are missing from the promoter sequence in Δ-66 compared to 150bp are required for E4BP4 to repress *dp5* promoter activity in these cells.

5.2.7 Overexpression of E4BP4 reduces the activity of a *dp5* reporter construct in the presence and absence of NGF

Although E4BP4 appears to repress the basal activity of *dp5* reporter constructs containing the E4BP4-binding site in PC6.3 cells it was important to establish whether a similar effect was seen in sympathetic neurons. Before testing the effect of overexpressing E4BP4 on a *dp5* reporter construct in these cells the E4BP4 expression

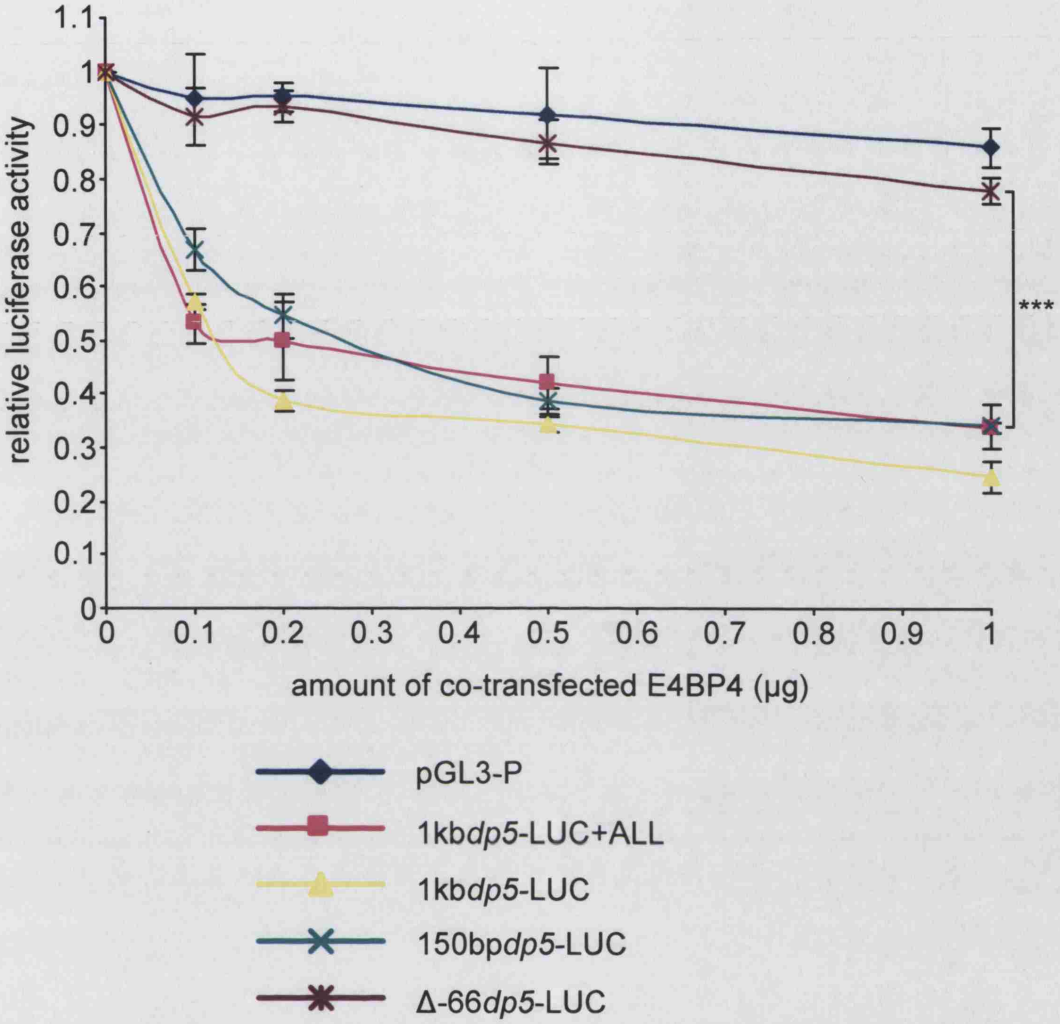


Figure 5.7 Dose response of *dp5* reporter constructs co-transfected with an E4BP4 expression vector into PC6.3 cells

Neuronally differentiated PC6.3 cells were transiently transfected with the luciferase reporter constructs pGL3-P, 1kb*dp5*-LUC+ALL, 1kb*dp5*-LUC, 150bp*dp5*-LUC and Δ-66*dp5*-LUC. The constructs were transfected at equimolar amounts, with pGL3-P transfected at 0.1 µg of DNA per well. 0, 0.1, 0.2, 0.5 or 1µg of an E4BP4 expression vector was co-transfected and the total amount of co-transfected DNA was made up to 1 µg with empty pcDNA3.1. The *Renilla* luciferase construct pRL-TK was also transfected (0.05 µg per well). Luciferase activity was determined 24 hours after transfection using a dual luciferase assay. Firefly luciferase activity was normalised for *Renilla* luciferase activity and the activity of each construct was calculated relative to the activity with 1 µg of pcDNA3.1, which was set as 1. The results of 3 independent transfection experiments, each performed in duplicate ± the SEM are shown (*** $P<0.001$, *t*-test).

vector was microinjected into sympathetic neurons and then analysed by immunofluorescence to check for E4BP4 expression. Neurons were injected with both the E4BP4 expression vector and guinea pig IgG, which acted as an injection marker. After fixation, microinjected neurons were identified by staining with an antibody against guinea pig IgG and whether these injected cells expressed E4BP4 was determined by staining with an E4BP4 antibody. As shown in Figure 5.8, the microinjected cells did express E4BP4 and this localised to the nucleus. E4BP4 was clearly expressed in ~80% of the injected cells, suggesting that the expression vector was functioning as expected in sympathetic neurons.

The effect of overexpressing E4BP4 on the activity of the construct 1kb*dp5*-LUC+ALL in sympathetic neurons was tested by co-injecting this construct with either the empty vector pcDNA3.1 or the E4BP4 expression vector, as well as pRL-TK. These cells were maintained in the presence or absence of NGF for ~20 hours. After this time, luciferase activity was determined and firefly luciferase activity was normalised to *Renilla* luciferase activity. The activity in each condition was calculated relative to that in cells that had been microinjected with pcDNA3.1 and maintained in the presence of NGF (Figure 5.9). When NGF was present, overexpression of E4BP4 reduced the activity of the *dp5* reporter construct 1kb*dp5*-LUC+ALL relative to cells co-injected with an empty vector control (pcDNA3.1). Following NGF deprivation, 1kb*dp5*-LUC+ALL co-injected with pcDNA3.1 responds in a similar manner as to when this construct is injected alone since the relative luciferase activity increases ~4.5 fold. However, this activity is significantly reduced when E4BP4 is overexpressed. Although this activity is an induction of ~4.2 fold relative to the activity when E4BP4 is overexpressed in the presence of NGF, the level after NGF withdrawal is less than the basal expression of this construct when injected with pcDNA3.1. These results indicate that, as in PC6.3 cells, overexpression of E4BP4 reduces the activity of a *dp5* reporter construct that contains the E4BP4-binding site, and that this repression occurs in both the presence and absence of NGF.

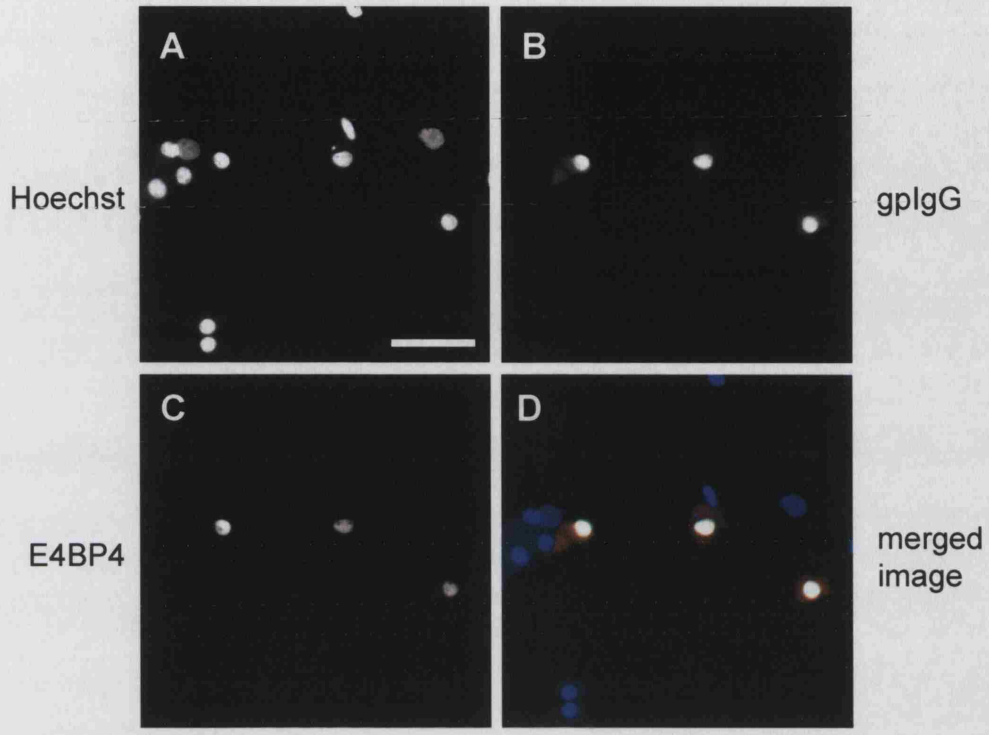


Figure 5.8 Localisation of E4BP4 in sympathetic neurons microinjected with an E4BP4 expression vector

An example of sympathetic neurons microinjected with an E4BP4 expression vector and then analysed by immunocytochemistry. Sympathetic neurons were microinjected with E4BP4 [0.05 μg/μl] together with gpIgG [2.5 μg/μl] and were maintained for 20-24 hours before immunocytochemistry was performed. **A)** Hoechst staining of all nuclei, **B)** guinea pig IgG staining identifies microinjected cells, **C)** cells stained with anti-E4BP4 polyclonal antibody indicate that E4BP4 is expressed, **D)** merged image of all three colours. The bar represents 60 μm.

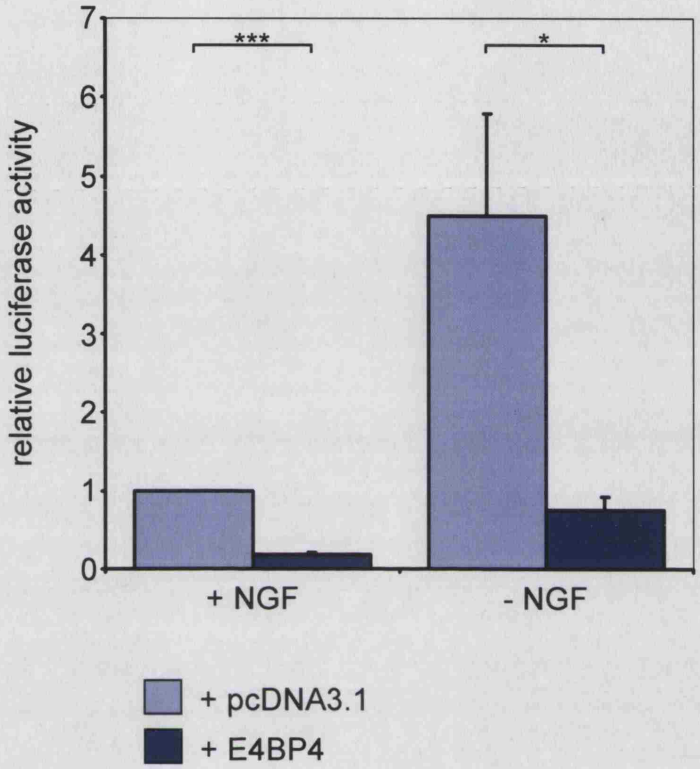


Figure 5.9 Effect of E4BP4 on the activity of a *dp5* reporter construct in sympathetic neurons cultured in the presence and absence of NGF

Sympathetic neurons were microinjected with 1kb*dp5*-luc+ALL [0.01 μg/μl], together with a *Renilla* luciferase expression vector [0.005 μg/μl], and either pcDNA3.1 or an E4BP4 expression vector [0.02 μg/μl]. Cells were maintained for ~20 hours +/- NGF and after this time luciferase activity was determined by using the dual luciferase assay. Firefly luciferase activity was normalised for *Renilla* luciferase activity and the firefly luciferase activity was calculated relative to activity +NGF with pcDNA3.1. The mean of 5 experiments ± the SEM is shown (* $P<0.05$, *** $P<0.001$, *t*-test).

5.2.8 Mutating the E4BP4-binding site in the *dp5* promoter abolishes repression by E4BP4

To test whether the repression of *dp5* promoter activity by E4BP4 occurs through the interaction of E4BP4 and the binding site identified in the promoter, the effect of mutating this sequence was examined. The three point mutations that were shown to abolish the binding of E4BP4 to the *dp5* sequence (Figure 5.6) were introduced into the *dp5* reporter construct that contained the 1 kb promoter sequence, intron and 3'UTR sequences by site-directed mutagenesis (1kb*dp5*-LUC+ALLmut). Whether these mutations were sufficient to prevent the repression of luciferase activity by E4BP4 was initially determined by the transfection of PC6.3 cells as described above (Section 5.2.6). The effect of overexpressing E4BP4 in cells co-transfected with either pGL3-P, 1kb*dp5*-LUC+ALL or 1kb*dp5*-LUC+ALLmut was tested in a dose response experiment. Both pGL3-P and 1kb*dp5*-LUC+ALL responded as previously, however the luciferase activity of 1kb*dp5*-LUC+ALLmut, which differs from 1kb*dp5*-LUC+ALL by 4 bases, is not repressed by increasing amounts of E4BP4 and follows a similar pattern of activity as the pGL3-P negative control (Figure 5.10). This suggests that repression by E4BP4 occurs via the binding of E4BP4 to the identified site in the *dp5* promoter.

5.2.9 Mutating the E4BP4-binding site reduces *dp5* reporter construct activity in the presence and absence of NGF

Since mutating the E4BP4-binding site in the *dp5* promoter eliminates the repressive effect of E4BP4 on basal promoter activity, the effect of mutating this site on the response to NGF deprivation was examined. The wild type construct 1kb*dp5*-LUC+ALL and the reporter construct containing the mutations that eliminate E4BP4 binding, 1kb*dp5*-LUC+ALLmut, were microinjected into sympathetic neurons with the *Renilla* luciferase construct pRL-TK. After microinjection, the neurons were maintained + or – NGF for ~20 hours. Luciferase activity was determined using a dual luciferase assay and firefly luciferase activity was normalised for *Renilla* luciferase activity. The luciferase activity in each condition was calculated relative to the activity in cells that had been microinjected with 1kb*dp5*-LUC+ALL and maintained in the presence of NGF

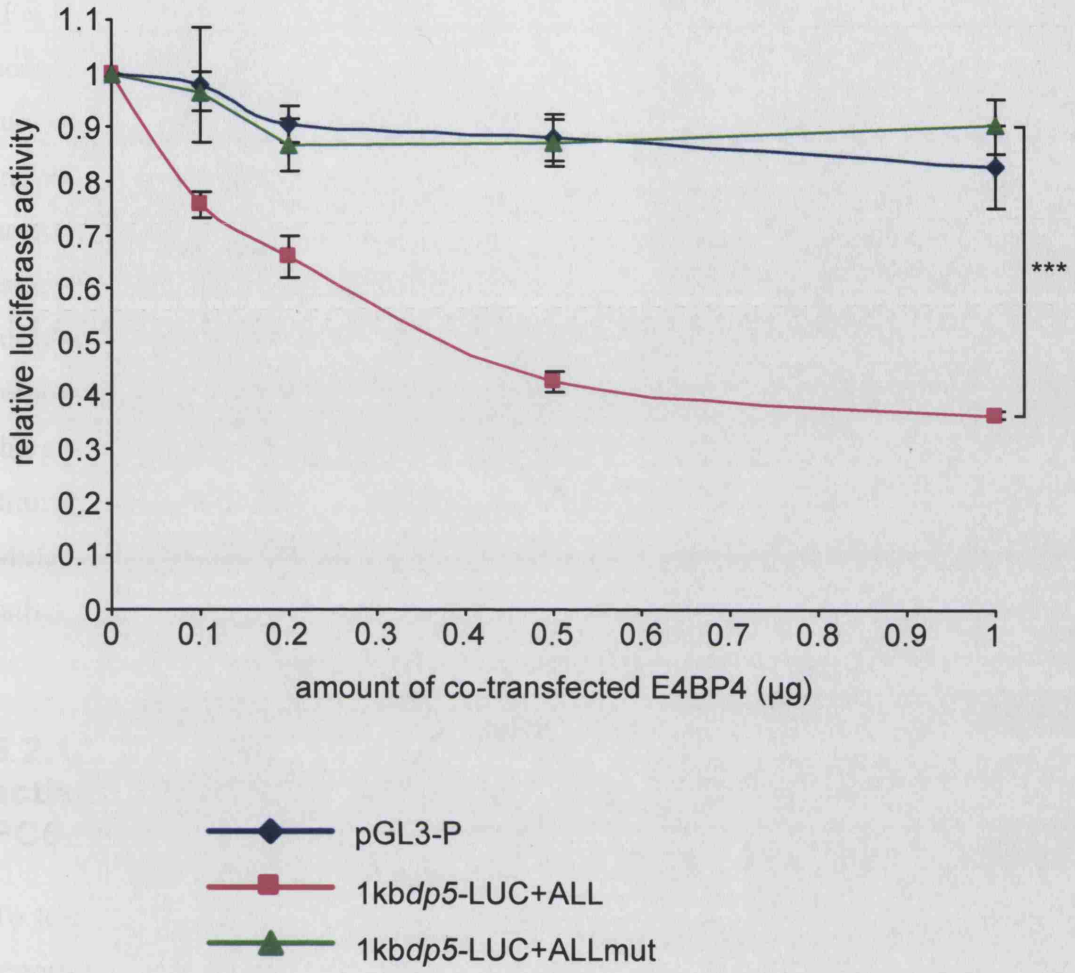


Figure 5.10 Dose response of a mutant *dp5* reporter construct co-transfected into PC6.3 cells with an E4BP4 expression vector

Neuronally differentiated PC6.3 cells were transiently transfected with the luciferase reporter constructs pGL3-P, 1kb*dp5*-LUC+ALL and 1kb*dp5*-LUC+ALLmut. The constructs were transfected at equimolar amounts, with pGL3-P transfected at 0.1 µg of DNA per well. 0, 0.1, 0.2, 0.5 or 1µg of an E4BP4 expression vector was co-transfected and the total amount of co-transfected DNA was made up to 1 µg with empty pcDNA3.1. The *Renilla* luciferase construct pRL-TK was also transfected (0.05 µg per well). Luciferase activity was determined 24 hours after transfection using a dual luciferase assay. Firefly luciferase activity was normalised for *Renilla* luciferase activity and the activity of each construct was calculated relative to the activity with 1 µg pcDNA3.1, which was set as 1. The results of 3 independent experiments, each performed in duplicate ± the SEM are shown (*** $P<0.001$, *t*-test).

(Figure 5.11). In agreement with the results obtained previously, the activity of the construct 1kb*dp5*-LUC+ALL increased after NGF deprivation (7.1 fold). Although the luciferase activity of 1kb*dp5*-LUC+ALLmut also increased after NGF withdrawal, induction was less than with the wild type construct (3.1 fold). Direct comparison of the activity of the wild type and mutant constructs in the same conditions indicates that the latter has a much lower activity. 1kb*dp5*-LUC+ALLmut shows a significant decrease in luciferase activity of 86% and 94% in the presence and absence of NGF respectively relative to 1kb*dp5*-LUC+ALL. This result suggests that eliminating E4BP4 binding to the site in the *dp5* promoter may decrease *dp5* induction after NGF deprivation but also dramatically reduces promoter activity in both the presence and absence of NGF. Mutating this site has the same effect as overexpressing E4BP4 which suggests that rather than binding a repressor, this site binds an activator in sympathetic neurons.

5.2.10 Mutation of the E4BP4-binding site reduces the basal activity of some, but not all, of the *dp5* reporter constructs in PC6.3 cells

To test the effect of mutating the E4BP4-binding site on the basal activity of other *dp5* reporter constructs containing only promoter sequences from the *dp5* gene, the mutations that eliminate binding were introduced into the constructs 1kb*dp5*-LUC and 150bp*dp5*-LUC (named 1kb*dp5*-LUCmut and 150bp*dp5*-LUCmut respectively). These constructs and 1kb*dp5*-LUC+ALLmut, as well as the equivalent wild type controls, Δ-66*dp5*-LUC, Δ-51*dp5*-LUC and pGL3-basic, were transiently transfected into PC6.3 cells. Luciferase activity was determined 24 hours after transfection by a dual luciferase assay and following normalisation against *Renilla* luciferase activity, firefly luciferase activity was calculated relative to the activity of the empty vector pGL3-basic (Figure 5.12). The activity of the wild type *dp5* reporter constructs was comparable to the results obtained previously since 1kb*dp5*-LUC+ALL had a substantially reduced basal activity compared to 1kb*dp5*-LUC (91% decrease) and 150bp*dp5*-LUC, although shorter than 1kb*dp5*-LUC, was as active. Δ-66*dp5*-LUC and Δ-51*dp5*-LUC also showed the same activities as previously with the activity significantly dropping with the sequential deletion of the promoter sequence ($p<0.001$). Both 1kb*dp5*-LUC+ALLmut and 1kb*dp5*-

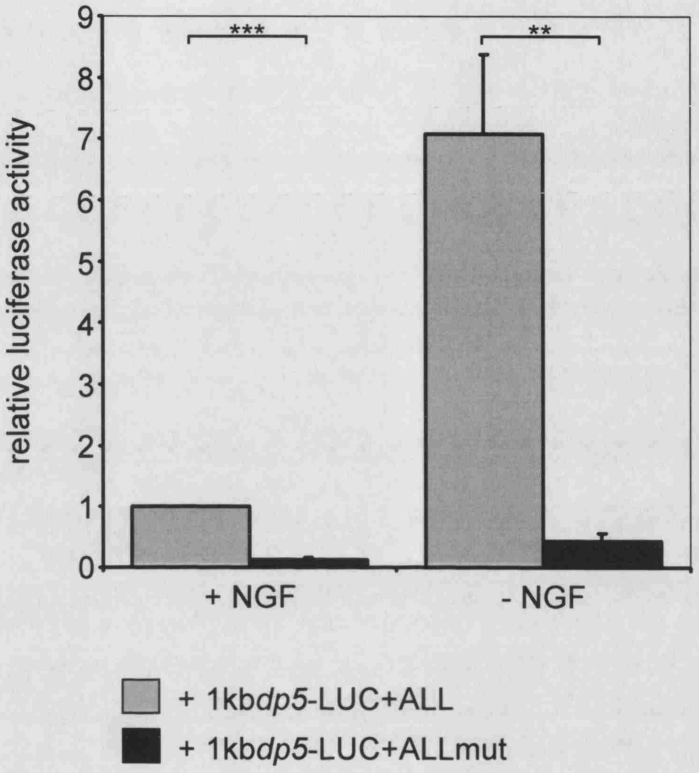
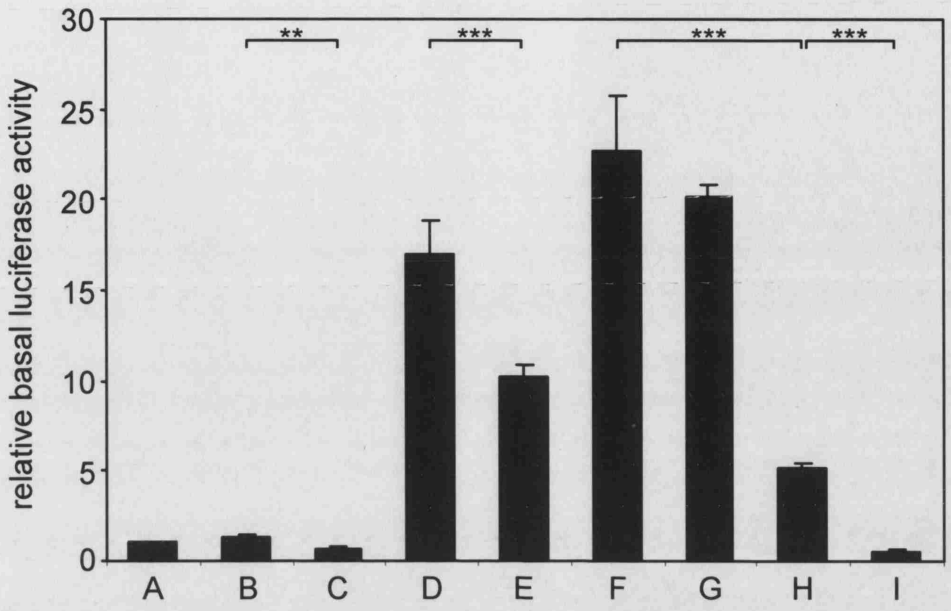


Figure 5.11 Response of a mutated *dp5* reporter construct to NGF deprivation after microinjection into sympathetic neurons

Sympathetic neurons were microinjected with 1kb*dp5*-luc+ALL or 1kb*dp5*-LUC+ALLmut [0.01 µg/µl], together with a *Renilla* luciferase expression vector [0.005 µg/µl]. Cells were maintained for ~20 hours +/- NGF and after this time luciferase activity was determined by using the dual luciferase assay. Firefly luciferase activity was normalised for *Renilla* luciferase activity and the firefly was calculated relative to activity +NGF with 1kb*dp5*-LUC+ALL, which was set at 1. The mean of 6 experiments ± the SEM is shown (** $P<0.01$, *** $P<0.001$, *t*-test).



A = pGL3-basic	F = 150bp*dp5*-LUC
B = 1kb*dp5*-LUC+ALL	G = 150bp*dp5*-LUC+mut
C = 1kb*dp5*-LUC+ALLmut	H = Δ-66*dp5*-LUC
D = 1kb*dp5*-LUC	I = Δ-51*dp5*-LUC
E = 1kb*dp5*-LUC+mut	

Figure 5.12 Comparison of the basal activity of *dp5* luciferase reporter constructs containing mutated E4BP4-binding sites

Neuronally differentiated PC6.3 cells were transiently transfected with luciferase reporter constructs containing mutated E4BP4 binding sites, and the corresponding wild type construct. The empty vector pGL3-basic was also transfected as a control. The constructs were transfected at equimolar amounts, with pGL3-basic transfected at 0.1 μg of DNA per well. The *Renilla* luciferase construct pRL-TK (0.05 μg per well) was also transfected. Luciferase activity was determined 24 hours after transfection using a dual luciferase assay. Firefly luciferase activity was normalised for *Renilla* luciferase activity and the activity of each construct was calculated relative to pGL3-basic, which was set as 1. The results of at least 3 independent experiments, each performed in duplicate ± the standard error of the mean (SEM) is shown (** $P<0.01$, *** $P<0.001$, *t*-test).

LUCmut show significantly decreased basal activities of 42% and 37% respectively compared to the equivalent wild type constructs ($p<0.01$). Mutating the E4BP4 site in the construct 150bp*dp5*-LUCmut does not appear to have a large effect on basal promoter activity relative to the wild type sequence and does not have the same effect as deleting the 36 bp containing this site, as shown by the 75% reduction in the promoter activity of Δ-66*dp5*-LUC compared to 150bp*dp5*-LUC. This result suggests that mutating the E4BP4-binding site in the *dp5* promoter causes a decrease in the basal activity of some but not all *dp5* reporter constructs. In addition, the decrease observed by mutating this sequence is less than the effect of deleting the whole of the region containing the E4BP4-binding site.

5.3 Discussion

In the experiments described in this chapter I investigated the contribution of different regions of the *dp5* gene to basal promoter activity and the *dp5* minimal promoter was defined. Potential transcription factor binding sites were identified within a short region of the promoter, including an E4BP4-binding site, which was shown to be able to bind E4BP4 *in vitro*. The potential role of this site and of E4BP4 in *dp5* regulation was investigated and the results suggested that although E4BP4 was able to repress *dp5* promoter activity, this site may actually be bound by an activator in sympathetic neurons.

Comparison of the basal activity of *dp5* reporter constructs that had been previously tested for a response to NGF deprivation in both PC6.3 cells and sympathetic neurons indicated that the addition of some sequences had a pronounced effect on basal promoter activity. The results obtained suggested that the constructs behaved differently in the different cells types since the presence of the 3'UTR repressed promoter activity in PC6.3 cells but not sympathetic neurons, whereas the intron sequence reduced the activity in sympathetic neurons but not PC6.3 cells. This highlights one of the problems of using the PC6.3 cell system as a substitute for sympathetic neurons since the results obtained are not always comparable. However the construct 1kb*dp5*-LUC+ALL, which contained all three of the major regions of the *dp5* gene, showed low basal activity in

both systems relative to the promoter alone, which may reflect more accurately the activity of the promoter in its normal context. This result demonstrates that the PC6.3 cell system can be useful for studying the behaviour of some reporter constructs but also highlights the importance of ensuring that any model cell line used is a good representative of the primary cells. The combined results indicated that in the presence of the intron and the 3'UTR the basal activity of the *dp5* promoter is reduced to a low level, suggesting that sequences from within these regions may be having a repressive effect on promoter activity.

Alone, 1 kb of the sequence from upstream of the *dp5* transcriptional start site appeared to have a reasonably high basal activity, therefore closer examination of the promoter was performed in an attempt to localise any potentially interesting sequences that may be involved in regulating *dp5* promoter activity. Sequentially deleting the promoter sequence at the 5' end was a suitable approach for initially identifying any promoter regions that appeared to have a large effect on promoter activity and this strategy has been successfully used in transfected PC12 cells to identify regions involved in regulating the activity of the neuropeptide precursor VGF and β-amyloid precursor protein promoters (Canu *et al.*, 1997;Lahiri *et al.*, 2000). It was important that reporter constructs were transfected at equimolar amounts to ensure that a similar copy number of each plasmid was transfected since some constructs varied considerably in size. When comparing the activity of different lengths of promoter sequence there did not appear to be large differences in the activity of sequences ranging from 150 bp to 4.5 kb although the results did indicate that a sequence between 1.6 and 4.5 kb upstream of the transcriptional start site could contain a region that represses promoter activity. It was interesting to discover that a relatively short region of the promoter had the same activity as a much longer sequence but deletion of just 51 base pairs reduced activity to the level of the promoterless vector pGL3-basic. Deletion of only 36 bases including the E4BP4-binding site reduced promoter activity but not to the same extent as the larger deletion which indicates that the extra 15 bp region including the GC box contains sequences that increase promoter activity. Since the AT-rich sequence is situated too far upstream for a classical TATA box, the *dp5* promoter is TATA-less. Promoters lacking TATA boxes often contain GC-rich regions that are important for transcription

initiation. For example, the cyclin-dependent kinase 5 regulator p35 has a TATA-less promoter that contains a GC-rich region, which also includes a GC box. This has been shown to be critical for promoter activity in neurons (Ross *et al.*, 2002) and it is possible that the *dp5* GC box is also a key element for promoter activity. The effect of deleting the other identified potential sites cannot be established since the activity of the promoter is negligible after removal of the GC box. Therefore to determine whether any of these sites play a role in regulating promoter activity, mutations that abolish binding should be introduced into either 150bp*dp5*-LUC or Δ-66*dp5*-LUC so that the effect of preventing interaction with these sites can be determined. In addition, the activity of these different length promoters should be confirmed in sympathetic neurons to ensure that any decrease in activity is reproduced in these cells.

Identifying a potential E4BP4-binding site within the *dp5* proximal promoter was of particular interest as this transcription factor has been shown to be expressed during motoneuron development and overexpression of E4BP4 inhibits apoptosis induced by trophic factor withdrawal in these neurons (Junghans *et al.*, 2004). *dp5* also appears to be important for apoptosis in this cells type since motoneurons from $dp5^{-/-}$ mice are partially protected from axotomy-induced death (Imaizumi *et al.*, 2004). It was essential to establish whether E4BP4 was expressed in sympathetic neurons and the results indicate that although *e4bp4* mRNA is expressed in this cell type the levels do not appear to be affected by NGF withdrawal. The level of *e4bp4* mRNA in both the presence and absence of NGF followed the same pattern of expression at different time points after the change in media at 0 hours, with levels increasing slightly after 24 hours. Although there does appear to be a degree of variation, reflected by the large error bars, it is possible that changing the medium *per se* could affect *e4bp4* expression. This result does not exclude the possibility that E4BP4 could contribute to *dp5* regulation by NGF deprivation as it is possible that protein levels could alter or post-translational modifications could occur in different conditions. For example, E4BP4 can be phosphorylated and there is evidence that this can alter its DNA binding activity (Chen *et al.*, 1995). In motoneurons E4BP4 protein and mRNA is not regulated by trophic factor withdrawal but has been found to be developmentally regulated, with cells that survive periods of naturally occurring cell death having the highest level of expression

(Junghans *et al.*, 2004). *e4bp4* expression during the development of sympathetic neurons could be examined in a similar way by isolating neurons from the superior cervical ganglia of rats of different ages, for example P1, P7, P14 and P21 rats, to establish whether differences in expression could be seen at different stages of development and at times with varying levels of cell death.

Although the identified potential E4BP4-binding site matched the consensus sequence it was important to determine whether E4BP4 could actually bind to this site in the context of the *dp5* promoter. The result from the EMSA indicates that E4BP4 can bind specifically to this sequence and binding can be abolished by the introduction of three point mutations. The three point mutations were chosen because these changes have previously been shown by Cowell *et al* (1992) to individually decrease the relative binding affinity of the sequence to E4BP4 and by combining all three mutations I hoped to completely abolish any binding. A polymorphism was also introduced into the mutated sequence in which a thymine was substituted with a cytosine. This change should not have affected the binding ability of E4BP4 since the consensus sequence for an E4BP4-binding sites can be either T or C at this position and this change was introduced so that the mutated sequence matched that previously published. In addition to the band indicating the binding of E4BP4 to the wild type sequence, a non-specific band appeared in all lanes. It is likely that this is due to a non-specific factor arising during *in vitro* translation since this was present in samples containing no E4BP4, but unprogrammed lysate (pcDNA3.1 control).

A range of constructs were screened in co-transfection experiments in PC6.3 cells with an E4BP4 expression vector, kindly provided by Dr. J. Yeung (University College London), including the construct Δ-66*dp5*-LUC that lacked the region containing the E4BP4-binding site. A dose response to E4BP4 was measured but the total amount of co-transfected DNA was kept constant using the empty vector pcDNA3.1. The results suggested that E4BP4 represses *dp5* promoter activity when overexpressed, and repression is likely to occur via the identified site since promoter activity did not decrease in the construct without the region containing this site. The involvement of this site in repression was confirmed following the introduction of the mutations that prevent E4BP4 binding. The activity of the construct 1kb*dp5*-

LUC+ALLmut containing the mutations was not repressed by overexpression of E4BP4, suggesting that E4BP4 represses promoter activity via the identified site. These experiments emphasise an advantage of this cell system as a number of reporter constructs were tested with a range of amounts of E4BP4 in a single experiment, something which would not be possible if testing by microinjection into sympathetic neurons.

However, it is essential that after initial screening in PC6.3 cells interesting results should be confirmed in sympathetic neurons. After establishing that E4BP4 was expressed following microinjection of an expression vector into sympathetic neurons, the effect of overexpressing E4BP4 on the construct 1kb*dp5*-LUC+ALL was determined in the presence and absence of NGF in these cells. This construct appears to have a similar response to E4BP4 in sympathetic neurons compared to PC6.3 cells because the luciferase activity of 1kb*dp5*-LUC+ALL in the presence and also in the absence of NGF decreased following overexpression of E4BP4. Luciferase activity in cells co-injected with the E4BP4 expression vector did increase after NGF withdrawal, although this level of activity was lower than the basal activity of 1kb*dp5*-LUC+ALL in the absence of E4BP4. This suggests that any increase in expression after NGF deprivation may still be lower in the presence of E4BP4 than the basal levels of expression normally found in sympathetic neurons. These combined results demonstrated that E4BP4 can repress the activity of the *dp5* promoter in both PC6.3 cells and sympathetic neurons and, in PC6.3 cells, the repression occurs via the identified E4BP4-binding site. The effect of mutating this site was tested in sympathetic neurons. The result obtained following the microinjection of 1kb*dp5*-LUC+ALL and the mutated construct 1kb*dp5*-LUC+ALLmut into sympathetic neurons indicates that although E4BP4 can repress the activity of the *dp5* promoter, it is unlikely that this is occurring in sympathetic neurons because mutating this site to prevent E4BP4 binding has the same effect as overexpressing E4BP4 in both the presence and absence of NGF. A similar, but less dramatic response to mutating the E4BP4-binding site in a range of constructs was observed in PC6.3 cells, although mutating the site in a construct containing only 150 bp of the promoter did not appear to have a major affect on activity. The results obtained with these other constructs should be confirmed by microinjection into sympathetic neurons because

results using the two different systems are not always comparable, demonstrated by the basal activity of constructs containing different regions of the *dp5* gene as described above. If mutating this site does not cause the drop in promoter activity that occurs when the whole of this region is deleted, as in Δ-66*dp5*-LUC, this would suggest that this region may contain another sequence that increases basal promoter activity.

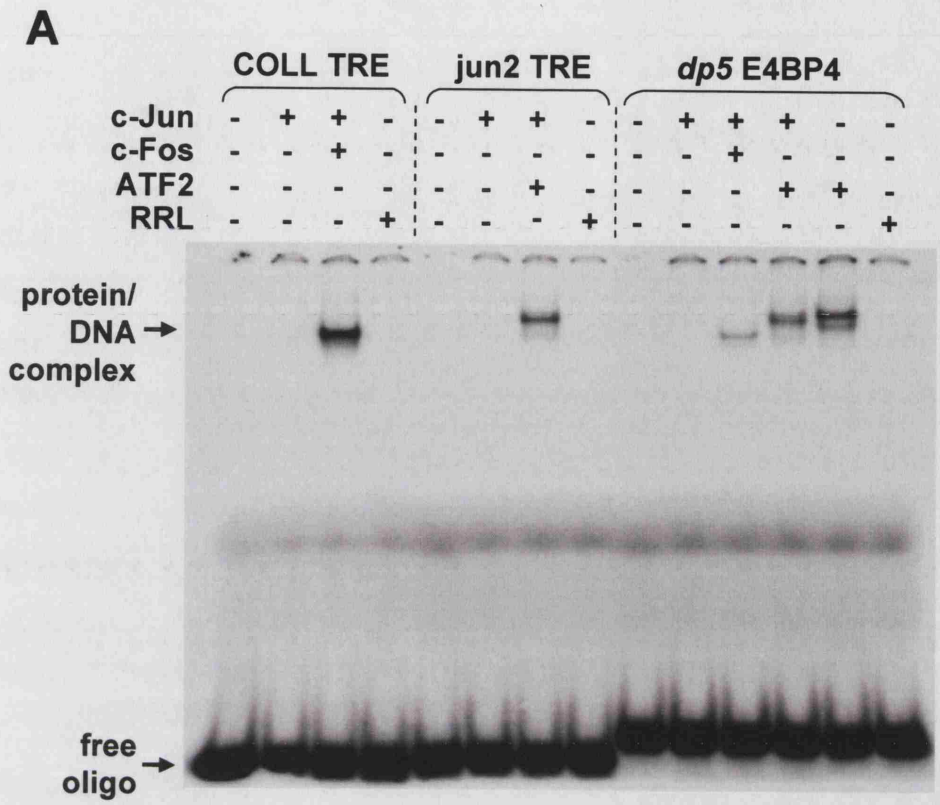
Combined, these results suggest that the E4BP4 site, rather than binding the repressor E4BP4, may be bound by an activator since mutation decreases promoter activity, as could occur if an activator was no longer able to bind. E4BP4 is closely related to the bZIP proteins HLF (hepatic leukaemia factor), DBP (D-box binding protein) and TEF (thyrotroph embryonic factor), which form the PAR (proline- and acidic- amino acid-rich) subfamily of proteins and all act as transcriptional activators (Mitsui *et al.*, 2001). E4BP4 lacks a PAR domain but the DNA binding domain is similar to that of HLP, DBP and TEF (Cowell, 2002) and, although E4BP4 is unable to form heterodimers with these PAR subfamily proteins, it can compete for the same binding sites (Mitsui *et al.*, 2001).

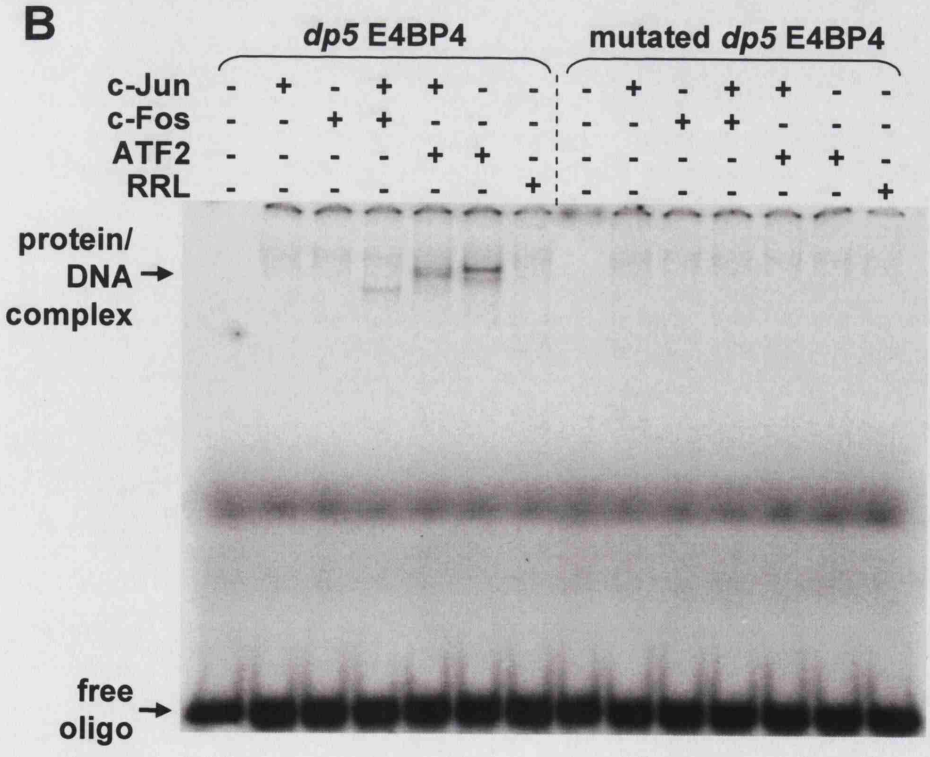
Alternatively, because this site has similarity to a CRE it is possible that this sequence in the *dp5* promoter could bind AP-1 family members, which can function as transcriptional activators. The ability of members of this family of transcription factors to bind to the *dp5* promoter was examined in our laboratory by EMSA in experiments performed by Dr. Jonathan Ham (Figure 5.13). The proteins c-Jun, c-Fos and ATF-2 were translated *in vitro* and their ability to bind to the E4BP4-binding site in the *dp5* promoter was analysed. Successful translation and the appropriate binding conditions were confirmed by testing the binding of these transcription factors to known AP-1 target sequences (Figure 5.13A). The collagenase promoter contains a TRE known to bind c-Jun/c-Fos heterodimers (van *et al.*, 1998) and analysis showed that protein/DNA complexes formed when radiolabelled oligonucleotides corresponding to the collagenase TRE were incubated with both c-Jun and c-Fos but not c-Jun alone. In parallel, oligonucleotides corresponding to the jun2 site from the *c-jun* promoter, known to bind c-Jun/ATF-2 heterodimers, appeared to bind c-Jun only when ATF-2 was also present. The binding of combinations of these AP-1 family proteins to the *dp5* E4BP4-binding site was tested using the oligonucleotide corresponding to this site (Figure 5.13A and B).

Figure 5.13 Binding of AP-1 family members to the *dp5* E4BP4-binding site

A) Radiolabelled double stranded oligonucleotides corresponding to the collagenase gene TRE, the *c-jun* promoter TRE jun2 and the *dp5* sequence containing the E4BP4-binding site were incubated with combinations of *in vitro* translated c-Jun, c-Fos and ATF2, or an empty vector control (RRL). The binding reactions were analysed by gel electrophoresis and visualised using phosphorimaging.

B) Radiolabelled double stranded oligonucleotides corresponding to the *dp5* sequence containing the E4BP4-binding site, as well as oligonucleotides containing three point mutations within this site, were incubated with combinations of *in vitro* translated c-Jun, c-Fos and ATF2, or an empty vector control (RRL). The binding reactions were analysed by gel electrophoresis and visualised using phosphorimaging.


A
COLL TRE
jun2 TRE
dp5 E4BP4
c-Jun
c-Fos
ATF2
RRL
protein/
DNA
complex
free
oligo


B
dp5 E4BP4
mutated dp5 E4BP4
c-Jun
c-Fos
ATF2
RRL
protein/
DNA
complex
free
oligo

A band, indicating the presence of protein/DNA complexes, was observed when the oligonucleotides had been incubated with c-Jun plus ATF-2, ATF-2 alone and c-Jun plus c-Fos, although the latter band was quite weak. Neither c-Jun nor c-Fos alone bound to the *dp5* sequence. Mutation of the E4BP4-binding site by the introduction of the 3 point mutations that abolished E4BP4 binding eliminated the binding of any of these transcription factors both as homo- or heterodimers (Figure 5.13B). These results indicate that transcription factors known to be activators can bind to the identified E4BP4-binding site in the *dp5* promoter although whether any of these activators play a role in regulating *dp5* activity in sympathetic neurons in response to NGF needs to be determined. In particular, it would be interesting to determine whether c-Jun is important for *dp5* regulation and whether this occurs via this identified site because c-Jun is a downstream target of the JNK/MLK pathway, which my previous results indicate is involved in regulating *dp5* levels (see Chapter 4).

Figure 5.14 illustrates a potential model for *dp5* promoter activation in which, although E4BP4 can bind to the *dp5* sequence, it is one of the related activators or a c-Jun/ATF-2 heterodimer that binds and activates transcription following NGF deprivation. Therefore future work should focus on these activators to identify whether any are involved in the upregulation of *dp5* expression following NGF withdrawal.

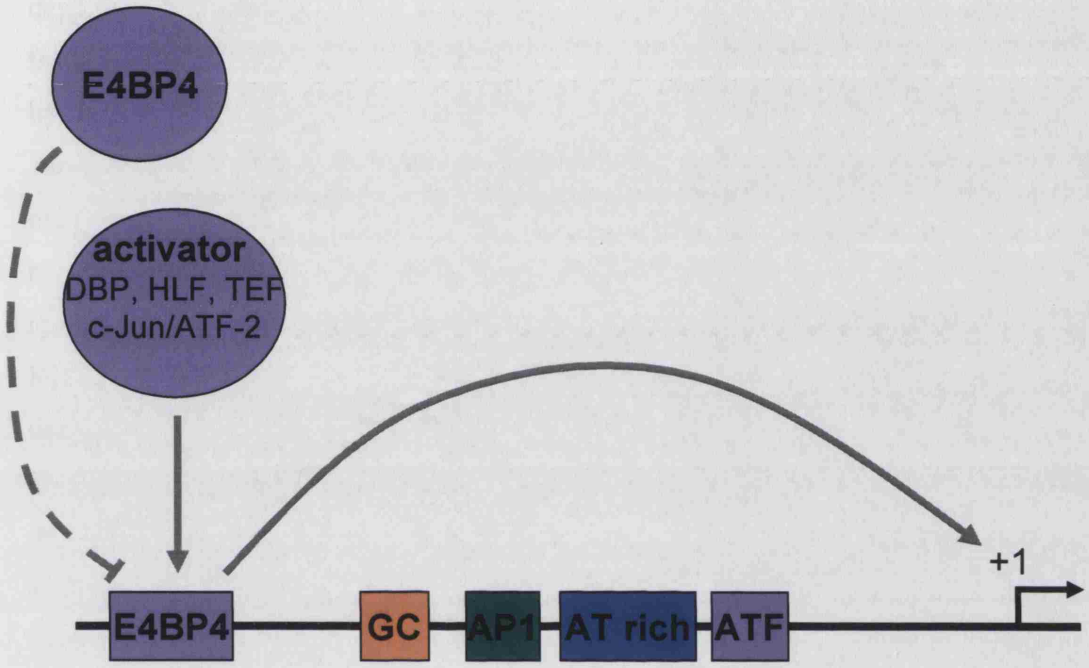


Figure 5.14 Model showing how the *dp5* promoter may be regulated via an E4BP4-binding site

Although E4BP4 can bind to the E4BP4 binding site found in the *dp5* promoter region and repress promoter activity, mutating this site strongly reduces basal *dp5* promoter activity, suggesting that this site binds an activator, possibly another PAR family member or c-Jun/ATF-2, that activates *dp5* transcription.

6 General discussion

Apoptosis plays an important role in normal physiology and is critical for the development of the nervous system. The survival of neurons during development depends on the presence of neurotrophic factors and those cells that receive insufficient trophic factors during this time die by apoptosis. This process can be modelled *in vitro* by culturing sympathetic neurons, which depend on nerve growth factor (NGF) for survival and which undergo apoptosis following the removal of NGF from the growth medium. Apoptosis in sympathetic neurons following NGF deprivation is tightly regulated and requires *de novo* gene transcription. Several proapoptotic BCL-2 family members are known to be induced in response to NGF withdrawal, including DP5, a BH3-only protein that was first identified as a gene that was transcriptionally upregulated in sympathetic neurons undergoing apoptosis triggered by NGF deprivation. Overexpression of DP5 induces apoptosis in the presence of NGF and the involvement of DP5 in this process is indicated by the analysis of sympathetic neurons from $dp5^{-/-}$ knockout mice, which are transiently protected from NGF withdrawal-induced apoptosis. The regulation of DP5 at the transcriptional level has been shown to involve the MLK/JNK pathway since the MLK inhibitor CEP-1347 reduces *dp5* mRNA induction in sympathetic neurons by approximately 75% after NGF deprivation. The precise mechanism by which this occurs is currently unknown.

To identify regions of the *dp5* gene that are involved in its regulation by NGF, I made luciferase reporter constructs that contained sequences from the promoter, either alone or in combination with sequences from the intron and/or the 3'UTR, and tested these by microinjection into sympathetic neurons. Luciferase induction following NGF withdrawal was determined and the results indicated that the presence of only 1 kb of sequence from upstream of the transcriptional start site was sufficient for the induction of luciferase activity in the absence of NGF. This induction was increased by the addition of ~400 bp from the intron that is conserved between the rat, mouse and human *dp5* genes and this appears to function as an enhancer in a context-specific manner. Addition of the 645 bp from the 5' end of the 3'UTR also resulted in a higher induction of luciferase activity after NGF deprivation, and this increase in activity appeared to

require sequences from both exon 1 and exon 2 of the *dp5* gene. It would be interesting to investigate whether this sequence affects the level of *dp5* expression by acting as an enhancer or by altering mRNA stability, since this could not be determined from the results of the experiments I performed. The reporter construct containing all three of the *dp5* regions showed the largest increase in luciferase activity after NGF withdrawal and had a similar level of induction as the endogenous *dp5* mRNA, as assessed by RT-PCR. This suggests that this construct responds to a similar extent as the *dp5* gene and may contain most, if not all of the regions that are involved in regulation by NGF.

In sympathetic neurons, MLK/JNK activity increases after NGF withdrawal, whereas PI3-K activity is inhibited. To study the role of these signalling pathways in regulating *dp5* mRNA levels in response to NGF, I tested the effect of altering the activity of the MLK/JNK and PI3-K pathways on the activity of different *dp5* reporter constructs. I performed experiments using three reporter constructs in which the MLK/JNK pathway was activated in the presence of NGF by overexpression of a MLK3 expression vector, or the MLK/JNK pathway was inhibited following NGF deprivation by treatment with the chemical compound CEP-11004 or PI3-K activity was reduced in the presence of NGF by treatment with LY294002. The results of these experiments suggested that the 1 kb promoter region, and possibly the intron, contain an element that responds to the MLK/JNK pathway to increase *dp5* expression following NGF withdrawal. In contrast to this, the sequence from the 3'UTR may contain a region that negatively regulates *dp5* expression in response to PI3-K activity that also prevents promoter activity following MLK/JNK pathway activation. This suggests that expression of *dp5* may not only require MLK/JNK activity but also inhibition of PI3-K, which could function to prevent inappropriate expression of this proapoptotic protein. Although I confirmed that inhibition of the MLK/JNK pathway by CEP-11004 reduced the induction of endogenous *dp5* after NGF withdrawal, the effect of inhibiting the PI3-K pathway using LY294002 was less conclusive and requires further investigation. It is interesting to note that the *dp5* 3'UTR contains a sequence conserved between rat and human that shows similarity to a FOXO-binding site. FOXO transcription factors are negatively regulated by the PI3-K/Akt pathway and have been shown to activate the transcription of the BH3-only family member *bim* (Gilley *et al.*, 2003). Therefore, if the

PI3-K pathway does regulate *dp5* levels, this site should be studied further. It would also be important to test the effect of altering the MLK/JNK and PI3-K pathways on the reporter construct containing all three regions of the *dp5* gene to examine how this construct behaves.

A crucial step in determining the elements involved in regulating transcription is the identification of the transcriptional start site. In the case of the *dp5* gene, this was performed using 5'RACE, a PCR-based technique that allows amplification from an mRNA template between a known internal site and an unknown mRNA end (Figure 6.1A). 5'RACE initially involves first strand cDNA synthesis from the mRNA template by reverse transcription, which is primed using a gene specific oligonucleotide. Following cDNA second strand synthesis, an adaptor molecule is added to the 3' end of the cDNA before a round of amplification using a gene specific primer and a primer specific for the 3' adaptor. The product of this second amplification can be cloned and sequenced to determine the 5' end of the mRNA. Sequencing of a number of different clones revealed that *dp5* only has one major transcriptional start site. This result is similar to that reported by Obata and colleagues (2003) who only observe one transcriptional start site for the human *hrk* gene, identified by comparison of genomic and cDNA sequences and which is only 2 base pairs downstream of the start site mapped for the rat *dp5* gene. 5' RACE is a useful method for mapping transcription initiation sites since it has the advantage of requiring only a small amount of mRNA as starting material. In addition to this, sequencing of cloned products allows for the precise sequence around the site to be obtained and this technique avoids the use of the radioactive probes that other methods rely on. Since 5'RACE involves the cloning of the 5'end of an mRNA, the resulting clones can be used for screening genomic libraries or in the construction of reporter genes.

To confirm our mapping of the rat *dp5* transcriptional start site, alternative methods for mapping transcriptional start sites could be used, such as an RNase protection assay or primer extension (Figure 6.1B & C). For both of these methods the general region of sequence containing the start site needs to be identified and can be established by nuclease protection or northern blot analysis using cloned restriction fragments as probes. The RNase protection assay involves hybridising a radioactively-

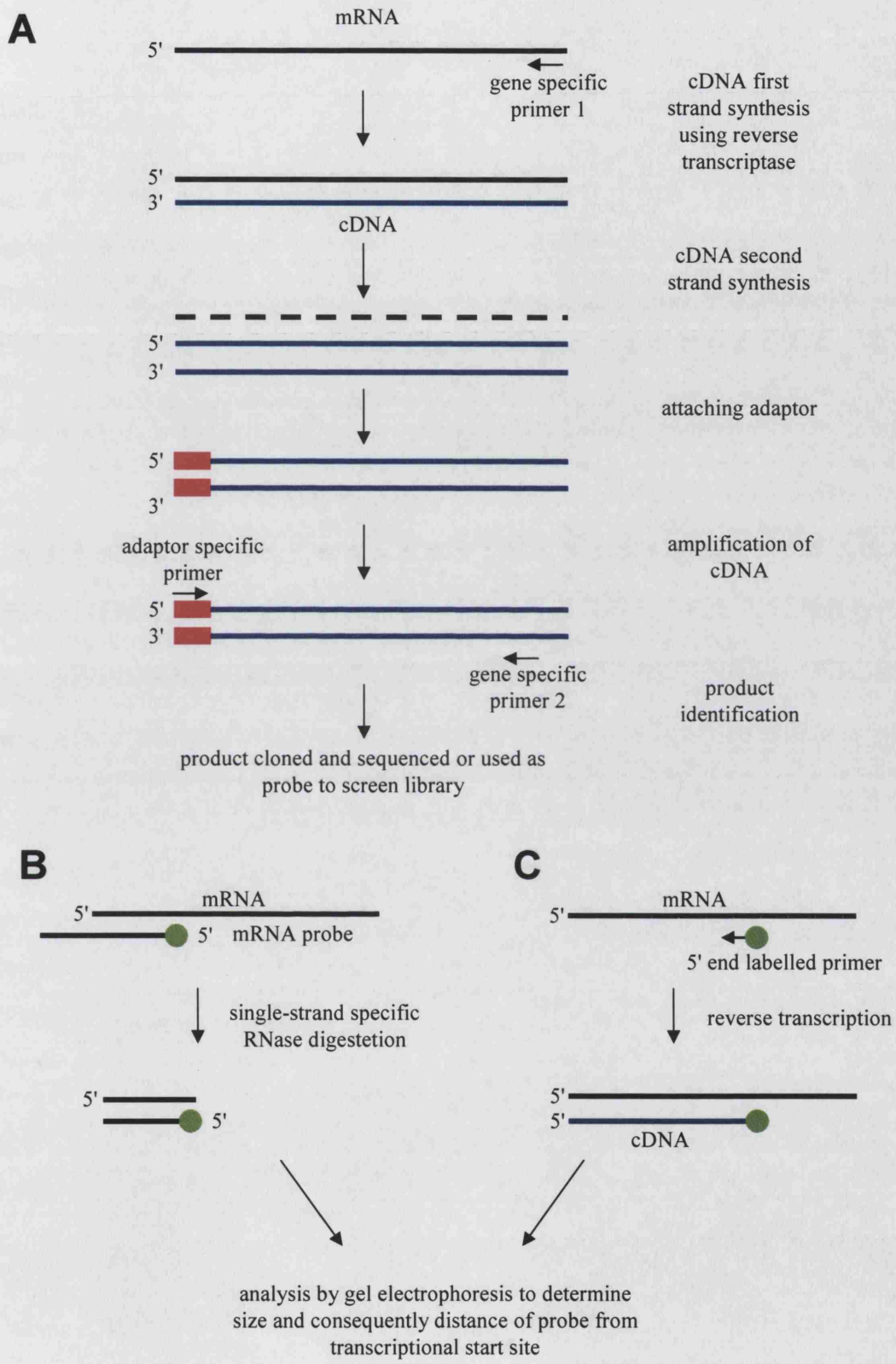


Figure 6.1 Outline of methods used for mapping transcriptional start sites

Transcriptional start sites can be mapped using 5' RACE **(A)**, as in the case of *dp5*, RNase protection assays **(B)** or primer extension **(C)**.

labelled RNA probe that extends beyond the region thought to contain the transcriptional start site to the target mRNA. The hybridised RNA is then digested with an RNase specific for single-stranded RNA. The paired strands are melted and the size of the digested labelled products determined by gel electrophoresis, which can be used to measure the 5' end of the mRNA and consequently the transcriptional start site. The primer extension method uses a 5' end-labelled oligonucleotide complementary to an internal region towards the 5' end of the mRNA sequence as a primer for DNA synthesis by reverse transcriptase, using the mRNA as a template. Since extension will stop at the end of the template the length of the resulting product can be analysed by gel electrophoresis and the position of the start site mapped. By comparing the primer extension product to a DNA sequencing ladder run in adjacent lanes the precise transcriptional start site can be mapped.

By identifying the precise transcriptional start site, analysis of the DNA sequences involved in transcription initiation can be performed, especially since many of the elements that form core promoters are situated at precise locations upstream or downstream of this site (reviewed in Smale, 2001;Smale and Kadonaga, 2003). TATA boxes are a common feature of core promoters and are located approximately 25 to 30 base pairs upstream of the transcriptional start site. Although these elements direct transcription from a predominant start site, TATA boxes are commonly associated with a cluster of 2 to 3 initiation sites that are grouped within 5 to 10 base pairs. The classic consensus sequence for this site is 5'-TATATAAG-3' although a wide variety of AT-rich sequences can interact with TATA-binding protein (TBP), part of the multisubunit TFIID transcription initiation complex, and consequently function as TATA boxes. An additional element, the TFIIB recognition element (BRE), can sometimes also be found in the region directly upstream of the TATA box. Although when initially identified it was thought that TATA boxes were common to all promoters, this is not the case as other core promoter elements are found either in conjunction with TATA boxes or in TATA-less promoters. One such element is the initiator (INR) element that can be found overlapping the transcriptional start site, from -3 to +5, and commonly has a cytosine at the -1 and adenine at the +1 positions. These elements are functionally similar to TATA boxes and are sufficient for accurate transcription initiation to occur

from a single start site, although they can act synergistically with TATA boxes. There is evidence that, like TATA boxes, INR elements can interact with TBP and other proteins such as RNA polymerase II have been reported to recognise the INR element. In TATA-less promoters, downstream promoter elements (DPE) are often found in conjunction with an INR element. These are located at precisely +28 to +32 relative to the transcriptional start site and typically function cooperatively with the INR element to bind TFIID. Although the above elements are all common to many promoters, not all promoters contain TATA boxes or INR elements. Such promoters are commonly GC-rich, often having long stretches containing these two bases, which are often recognised by the transcription factor Sp1, and contain multiple transcriptional start sites spread over 100 to 200 base pairs. A common feature of GC-rich promoters is the presence of high numbers of the dinucleotide CpG, a substrate for DNA methyltransferase, forming CpG islands. CpG islands normally lack a consensus sequence and appear to be independent of many of the core promoter elements described above. Methylation of cytosine to 5-methylcytosine in CpG islands results in repression of gene activity and is a possible mechanism for tissue-specific gene expression (Strachan and Read, 1999). It is possible that eukaryotes have such a wide variety of core promoters to allow a larger range of gene expression patterns from a limited number of transcription factors.

Examining the sequence of the *dp5* promoter indicates that this may be a TATA-less promoter since it lacks an AT-rich region 25 to 30 bases upstream of the transcriptional start site. An AT-rich region can be found between -30 and -43 but this may be too far upstream for a classic TATA box, although any possible role of this sequence as a core promoter element could be tested by either deleting or mutating this region. The *dp5* sequence was examined for the presence of a potential INR element and DPE, using the consensus sequences Py-Py-A-N-T/A-Py-Py and A/G-G-A/T-C/T-G/A/C respectively, but no potential sequences were found at the expected positions. Since the INR element consensus binding sequence allows for a degree of variation, it is possible that the region around the *dp5* start site may contain an INR element that varies from the above sequence by only two bases or this site could contain a sequence that binds other transcription factors. Identification of any potential INR element is of particular interest because this type of element is commonly associated with genes

containing a single transcriptional start site and only one start site has been identified for the *dp5* gene. This could be investigated by site directed mutagenesis to fully abolish any similarity to the consensus sequence INR to establish whether this region was important as a core promoter element, although in doing this, binding sites for other crucial elements could also be destroyed. Alternatively, the sequence around the transcriptional start site could be tested to determine whether this region could function as an INR element in the context of a heterologous gene. Examination of the *dp5* promoter sequence revealed the presence of a GC-box in the region upstream of the transcriptional start site and deletion of this site in a luciferase reporter construct reduced basal activity of the construct to that of the promoterless pGL3-basic. This indicates that in the *dp5* promoter the presence of a GC rich region may have an important role in core promoter activity. The human homologue *hrk* is known to contain a relatively large CpG island within the promoter that is involved in regulating gene expression and this site has been reported to be hypermethylated in certain human cancers (Obata *et al.*, 2003). Therefore, it is possible that GC-rich regions in the *dp5* promoter may have a similar function in regulating *dp5* gene expression.

Analysis of the 1 kb *dp5* promoter sequence used the experiments throughout this thesis identified some potential transcription factor binding sites upstream of the transcriptional start site. I tested the basal activity of reporter constructs containing sequential deletions of these potential sites by transient transfection into PC6.3 cells and these results suggested that the *dp5* minimal promoter was contained within a 150 bp sequence. A binding site for the transcription factor E4BP4 was identified, located at -93 upstream of the transcriptional start site. Although E4BP4 was shown to be able to bind to this site as well as repress the activity of *dp5* reporter constructs containing this sequence, it is unlikely that E4BP4 negatively regulates *dp5* expression in sympathetic neurons in response to NGF withdrawal because when the site was mutated to abolish E4BP4 binding it had the same effect on reporter construct activity as overexpression of E4BP4. The drop in promoter activity observed with the construct containing the mutated E4BP4-binding site indicates that an activator may bind to this site. Therefore an important line of study will be to identify any activators that can bind to this sequence in *dp5* and whether these are regulated by NGF in sympathetic neurons.

Some potential candidates are the related bZIP proteins HLP, DBP and TEF, which are activators that can compete for the same binding sites as E4BP4 (Mitsui *et al.*, 2001) and which are expressed in the nervous system (Gachon *et al.*, 2004). To determine any involvement in NGF-withdrawal induced apoptosis in sympathetic neurons, it would be important to determine whether these proteins are expressed in these cells and whether this is altered following the removal of NGF. This could be examined at the mRNA level by RT-PCR or at the protein level by western blotting if appropriate antibodies could be obtained. *dbp*$^{-/-}$, *hlf*$^{-/-}$ and *tef*$^{-/-}$ single, double and triple knockout mice are all anatomically normal, although triple knockout mice have a shortened lifespan, succumbing to epileptic seizures (Gachon *et al.*, 2004;Lopez-Molina *et al.*, 1997). Therefore it would be possible to examine *dp5* expression in neurons cultured from these animals to determine whether these proteins play a role in inducing *dp5*. In addition overexpression studies could be performed to establish whether these PAR family members function as activators to increase *dp5* levels and induce apoptosis in this system.

The E4BP4-binding site in the *dp5* promoter also showed high homology to an AP-1-binding site and the binding potential of combinations of AP-1 family members has been examined. These results indicate that c-Jun/c-Fos heterodimers, c-Jun/ATF-2 heterodimers and ATF-2 homodimers can bind to the sequence from the *dp5* promoter and mutating the site abolishes this interaction. This is an interesting result since *c-jun* expression and both c-Jun and ATF-2 phosphorylation increase in sympathetic neurons following NGF deprivation (Eilers *et al.*, 2001;Ham *et al.*, 1995) and activation of the *c-jun* promoter appears to occur via the binding of c-Jun/ATF-2 heterodimers to two TRE sites (Eilers *et al.*, 1998). One of these sites, the jun2 TRE (5'-TGACGTAA-3'), is only one base different to the *dp5* AP-1 site (5'-TGATGTAA-3'). Inhibition of AP-1 activity by microinjection of a dominant negative c-Jun (dn-Jun) lacking the c-Jun transactivation domain protects sympathetic neurons against NGF withdrawal-induced death, indicating the importance of AP-1 activity in this process (Ham *et al.*, 1995). In addition to this, a recombinant adenovirus expressing dn-Jun not only prevents cytochrome c release and reduces the number of apoptotic sympathetic neurons after NGF withdrawal, but also reduces the increase in mRNA levels of the BH3-only protein

BIM, indicating a requirement for c-Jun activity in the upregulation of proapoptotic *bim* (Whitfield *et al.*, 2001). The identification of a site within the *dp5* promoter that can bind c-Jun/ATF-2 is of particular interest since both c-Jun and ATF-2 are substrates for phosphorylation by JNK and I showed that a sequence in the promoter region responded to activation of the MLK/JNK pathway, although the specific involvement of JNK in *dp5* activity remains to be determined. This should be tested by direct inhibition of JNK through co-injection of the JNK-binding domain of the scaffold protein JIP-1 with the *dp5* reporter construct. The further investigation of the involvement of c-Jun/ATF-2 in *dp5* regulation would be interesting and to establish whether *dp5* is a target of c-Jun/ATF-2 activation, sympathetic neurons could be infected with a dn-Jun adenovirus. *dp5* mRNA levels after NGF withdrawal could then be measured by RT-PCR to determine whether induction was affected by c-Jun/ATF-2 inhibition. To examine the role of the AP-1-binding site within the *dp5* promoter, a dn-Jun expression vector could be co-injected with the *dp5* reporter construct containing all regions of the *dp5* gene to determine whether AP-1 inhibition prevents the activation of this construct in response to NGF deprivation. If so, the involvement of the identified binding site could be established by testing the effect of dn-Jun on the construct containing the mutated site. In addition, the effect of a dn-Jun expression vector that is unable to bind to ATF-2 on the *dp5* construct could also be tested to establish the involvement of ATF-2 in this process.

In addition to these studies, work could be performed to establish whether E4BP4 can function to regulate *dp5* in a different system. Overexpression of E4BP4 has been shown to protect motoneurons from apoptosis (Junghans *et al.*, 2004), a cell type that is also partially protected from axotomy-induced death by deletion of the *dp5* gene (Imaizumi *et al.*, 2004). Therefore, it could be interesting to investigate whether *dp5* can be negatively regulated by E4BP4 in motoneurons in response developmental or apoptotic stimuli such as axotomy or trophic factor deprivation. Additional studies could also be performed to try and identify what other proteins bind to the *dp5* gene and could involve a yeast one hybrid assay or DNA footprinting.

Reporter gene assays were used throughout this project to study the regions involved in regulating *dp5* expression in sympathetic neurons as well as some of the

signalling pathways involved in its regulation. Reporter genes are a useful system for the identification and characterisation of both promoters and enhancer elements, allowing the effect of both cis- and trans- acting factors to be examined (Schenborn and Groskreutz, 1999). There are many benefits to reporter gene assays as the effect of adding, deleting and mutating DNA sequences from the gene of interest can be determined, and this type of assay has been successfully used to identify regions that are important for the regulation of the BH3-only gene *bim* in response to NGF deprivation (Gilley *et al.*, 2003;Gilley and Ham, 2005). Despite this, there are some limitations to using reporter genes, which must be considered when analysing the results. Introducing reporter genes into cells results in the expression of DNA that is not normally present and it is always possible that other regions of the plasmid used to build the construct may have an effect, although this can be minimalised by carefully selecting the plasmid used. It is also possible that if high enough amounts of reporter construct are used squelching may occur in which the excess plasmid competes for and soaks up general transcription factors, affecting normal rates of transcription within the cell. This problem can be particularly apparent when overexpressing trans-acting factors since these can often be under the control of strong promoters. The arrangement of DNA within the reporter gene may also influence how it behaves, which could differ from the endogenous gene. In the reporter constructs 1kb*dp5*-LUC+3'UTR and 1kb*dp5*-LUC+ALL, which contain the *dp5* 3'UTR, sequences from both exon 1 and exon 2 are no longer separated by an intron and are arranged together. Due to its large size, it was not feasible to insert the full size intron fragment into a reporter construct and it is possible that this could affect the behaviour of these constructs since bringing these sequences together might result in the formation of new protein binding sites and so should be considered when concluding the results from experiments testing these constructs.

Another restriction of using reporter genes is that although they allow individual regions or combinations of specific regions to be examined, the results obtained may not truly represent the situation occurring with the endogenous gene. For example the reporter construct could lack regions that are essential for a component of transcription or control. This might be a sequence located away from the promoter that cannot be

easily identified and would result in the construct not behaving as the endogenous gene does. A major limitation to this type of assay relates to the structure of the DNA used. Although specific regions of the gene are introduced into the cell, the reporter gene lacks the chromatin structure of the endogenous gene. Chromatin structure plays an important part in altering gene expression and can be affected by a number of proteins that regulate transcription. Whether a gene is expressed is influenced by the degree of chromatin packaging as well as the position and nature of nucleosomes around regions required for transcription inititation both of which alter the accessibility of the DNA to transcriptional regulators such as TBP (reviewed in Bickmore, 2005;Mellor, 2005;Urnov and Wolffe, 2002).

Histone modification plays an important role in controlling gene expression and in particular histone acetylation is known to contribute to transcription activation. The attachment of acetyl groups to lysine residues reduces the affinity of histones for DNA and alters DNA packaging, allowing certain transcription factors to access their binding sites more easily. This process is mediated by histone acetyltransferases (HAT), some of which are transcriptional co-factors with a defined role in gene activation, for example p300/CBP (Ogryzko *et al.*, 1996). Many HATs also acetylate other targets, including the basal transcription machinery, indicating that these proteins may affect transcription through more than one mechanism. Conversely, some transcriptional repressors are histone deacetlyases (HDACs), such as the mammalian multiprotein complex Sin3 (Silverstein and Ekwall, 2005). This complex also contains CpG-binding proteins that bind to methylated DNA, suggesting that methylated CpG islands may be a target for HDAC attachment allowing the modification of chromatin to silence gene expression (Ng and Bird, 1999). Specific DNA-binding transcription factors play an important part in regulating the remodelling activities of both HAT and HDAC complexes because the binding of either transcriptional activators or repressors contributes to the recruitment of these histone modifiers. For example, the phosphorylation of CREB, which binds to CRE sequences, at serine 133 leads to the recruitment of the HAT CBP, resulting in transcriptional activation through both histone acetylation and its interaction with the basal transcription machinery (Johannessen *et al.*,

2004). In addition to acetylation, methyl and phosphate groups can also be added to histones, altering chromatin packaging and affecting gene expression.

Nucleosome remodelling also influences gene expression by altering chromatin structure allowing proteins access to bind DNA. This is an energy-dependent process that weakens nucleosome and DNA contact and can result in a change in nucleosome structure, cis-displacement along the DNA, or trans-displacement to a different region or second DNA. This process also involves large complexes that are thought to be recruited to target sites by interacting with DNA binding proteins. Some remodelling complexes have been shown to interact with HATs, suggesting that nucleosome remodelling may occur in conjunction with histone acetylation (Narlikar *et al.*, 2002).

Therefore, it is apparent that chromatin structure can have a significant role in regulating gene expression, something that cannot be tested by reporter gene assays but could play an important part in regulating the activity of the *dp5* promoter, especially since evidence suggests that histone deacetylation is associated with a loss in *hrk* expression in some cell lines (Obata *et al.*, 2003). If it was possible to stably transfect cells with the reporter gene being tested, the integrated plasmid DNA would be assembled into chromatin but the resulting chromatin structure would largely depend on the region of chromosomal DNA that the reporter gene was integrated into and may still lack the necessary surrounding sequences. To overcome this limitation, the effect of chromatin structure could be tested and any changes in structure under different conditions determined, for example in the presence and absence of NGF. It would also be important to compare the chromatin from *dp5* expressing and non-expressing cells, such as neurons versus fibroblasts, since differences between these cell types may be more apparent. Gross rearrangements in chromatin condensation such as movement and histone removal can be measured using a DNase hypersensitivity assay, in which DNA in an open and active state is identified due to its sensitivity to DNase digestion. In this assay, intact nuclei are exposed to limited DNase digestion before DNA isolation and restriction digestion. Following this, Southern blotting is performed using a specific probe that maps to one end of a restriction fragment containing the possible regulatory regions of the gene of interest. Comparison of DNase digested to non-digested samples aids the identification of additional shorter bands due to DNase activity. By determining

the size of these shorter band, the position of DNase hypersensitive sites relative to the restriction enzyme used can be mapped, which can be done more precisely through combining the results from different restriction enzyme digests. If DNase hypersensitive sites in the *dp5* gene were identified, it could be extrapolated that these were important for regulation, especially if this footprint was different in the presence and absence of NGF and activity was only determined in cells in which *dp5* is expressed relative to non-expressing cells. This method would aid the identification of important sites but in addition to this it is also essential to establish whether potential identified protein binding sites can actually bind the protein of interest *in vivo*, where chromatin structure could have a profound affect on binding ability. For example, although results indicate that the identified AP-1-like site in the *dp5* promoter can bind c-Jun/ATF2, it will be important to establish whether these proteins can actually bind to this sequence *in vivo* in neurons. Also, any alterations in the binding in the presence and absence of NGF should be measured. Chromatin immunoprecipitaion could be used to examine protein binding to the *dp5* promoter. This is performed by cross-linking the protein to DNA in living cells with formaldehyde before isolating the chromatin and shearing this into small fragments, typically between 200 and 1500 base pairs long. Immunoprecipitaion is performed against the protein of interest, for example c-Jun, before any cross-links between the protein and DNA are eliminated, the protein removed and then any bound DNA analysed by PCR using primers specific for the binding site being tested in the gene of interest.

Such experiments are necessary since although reporter gene assays are useful for identifying and testing important regions involved in gene regulation, it is critical that endogenous gene expression and the behaviour of the endogenous gene are determined, since this will more closely reflect the behaviour of DNA in the context in which it is normally found within a cell. This type of experiment should be performed although it is probably not possible to use sympathetic neurons because of the large amount of material required. As an alternative, chromatin structure in neuronally differentiated PC6.3 cells could be examined to give some indication of the structural behaviour of the endogenous *dp5* gene.

There is increasing evidence that apoptosis and the molecular pathways that lead to programmed cell death may play a role in various neurological disorders. For example, JNK signalling has been implicated particularly in Parkinson's disease (PD) and JNK deficiency can prevent neurodegeneration and improve motor function in an animal model of PD (Hunot *et al.*, 2004). Also, neuronal apoptosis induced by β-amyloid (Aβ), a protein implicated in the pathology of Alzheimer's disease, appears to require JNK activity and can be prevented by the MLK inhibitor CEP-1347, which also blocks Aβ-induced expression of *dp5* (Bozyczko-Coyne *et al.*, 2001;Troy *et al.*, 2001). Although phase II/III clinical trials of CEP-1347 as a treatment for PD have been terminated, the MLK/JNK signalling cascade still provides opportunity for therapeutic intervention. This indicates the importance of understanding the processes involved in regulating neuronal apoptosis, including the signalling cascades involved and the targets that are affected by these pathways. By increasing our knowledge of the mechanisms involved in inducing apoptosis, the role of this process in the pathology of disease and potential therapeutic targets may become apparent.

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